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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, C12N 15/00, A61K 37/00 — A1

(11) International Publication Number: WO 94/18345

(43) International Publication Date: 18 August 1994 (18.08.94)

(21) International Application Number: PCT/US94/01319

(22) International Filing Date: 4 February 1994 (04.02.94)

(30) Priority Data:
08/014,426 5 February 1993 (05.02.93) US
08/153,341 15 November 1993 (15.11.93) US

(71) Applicants: AFFYMAX TECHNOLOGIES N.V. [NL/NL]; De Ruyderkade 62, Curacao (AN). THE BOARD OF TRUSTEES OF THE LELAND STANFORD JR. UNIVERSITY [US/US]; Stanford, CA 94305 (US).

(72) Inventors: RENSCHLER, Markus, F.; 731 Alameda de la Pulgas, Redwood City, CA 941063 (US). LEVY, Ronald; 966 Mears Court, Stanford, CA 94305 (US). BHATT, Ramesh, R.; 777 West Middlefield Road, #18, Mountain View, CA 94043 (US). DOWER, William, J.; 761 Partridge Avenue, Menlo Park, CA 94025 (US).

(74) Agents: DUNN, Tracy, J. et al.; Townsend and Townsend Khourie and Crew, One Market Plaza, 20th Floor, Steuart Street Tower, San Francisco, CA 94105 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: RECEPTOR-BINDING ANTIPROLIFERATIVE PEPTIDES

(57) Abstract

Methods and composition are provided for identifying antiproliferative polypeptides which inhibit clonal expansion and/or induce apoptosis in cells of a predetermined cell population (e.g., a neoplastic cell sample) expression a cell surface receptor which is a member of the immunoglobulin superfamily. A predetermined cell population expressing surface immunoglobulin superfamily molecules is isolated from a patient as a cellular sample, such as a lymph node biopsy or blood sample containing neoplastic lymphocytic cells. Antiproliferative peptides which are identified by the methods of the invention can be used as therapeutic agents for treating lymphoproliferative disorders by anti-idiotype therapy.

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RECEPTOR-BINDING ANTIPROLIFERATIVE PEPTIDES

FIELD OF THE INVENTION

The invention provides novel polypeptides which can be used to treat neoplasia, lymphoproliferative diseases, and other pathological states, methods for identifying and administering such peptides or peptidomimetics for therapy and diagnosis of disease, methods of treating neoplastic disease, methods of treating immune diseases, and kits for identifying and producing therapeutic peptides for individual patients.

BACKGROUND

Signalling by membrane immunoglobulin, T cell receptors, and MHC molecules regulates lymphocyte maturation and development. For example, crosslinking of membrane immunoglobulin by antigen or by anti-immunoglobulin antibodies inactivates immature B cells, eliminating many of the B cells capable of producing autoantibodies; whereas crosslinking of membrane immunoglobulin promotes activation of mature B cells for clonal expansion and antibody production against foreign antigens. Activation of protein kinase activity and growth arrest of B cells by crosslinking of membrane immunoglobulin has been reported (Gold et al. (1990) Nature 345: 810; Campbell and Sefton (1990) EMBO J. 9: 2125). Anti-membrane immunoglobulin antibodies have been reported to exert inhibitory effects on cell proliferation of immature B cells, possibly by inducing programmed cell death, referred to as apoptosis (Benhamou et al. (1990) Eur. J. Immunol. 20: 1405). Apoptosis of cytotoxic T lymphocyte clones incubated in the presence of their specific epitopes (i.e., which bind to their surface T cell receptor idiotype) but not in the presence of irrelevant epitopes has also been reported (Moss et al. (1991) J. Exp. Med. 173: 681). Recently, crosslinking of the APO-1 cell surface antigen which is expressed on the surface of activated human T and B

lymphocytes has been reported to result in programmed cell death (Oehm et al. (1992) J. Biol. Chem. 267: 10709). The PD-1 gene, which is reportedly a member of the immunoglobulin gene superfamily, has been reported to be induced during programmed cell death of both a murine T cell hybridoma and a murine hematopoietic progenitor cell line (Ishida et al. (1992) EMBO J. 11: 3887).

The surface Ig receptor of B-cell lymphomas is a tumor specific antigen unique to each patient. Antibodies raised against the Ig receptor idiotype ("anti-Id") reportedly can arrest the growth of lymphoma cells in vitro (Pennell C and Scott D Eur J Immunol 16: 1577) and reportedly can induce tumor regression in vivo (Maloney et al. (1992) Biologic Therapy of Cancer Updates Vol. 2, No. 6, pp. 1-9; Miller et al. (1982) N Engl J Med 306:517). The anti-proliferative effect of anti-idiotype antibodies has been reported to involve transmembrane signalling through phosphoinositide hydrolysis, increased intracellular calcium, protein kinase C activation, and tyrosine phosphorylation (DeFranco AL (1992) Eur J. Biochem 210:381; Campbell MA and Sefton BM (1990) EMBO Journal 9:2125; Gold et al. (1990) Nature 345:810). However, the clinical utility of monoclonal anti-idiotypic antibodies has been limited primarily by the difficulty in producing custom antibodies for each patient. In addition, foreign antibodies (e.g., murine antibodies, chimeric antibodies, and the like) are immunogenic in human patients and frequently engender significant immune responses (e.g., human anti-murine antibody response; HAMA) making them unsuitable for long-term therapeutic use.

Despite these reported findings, rational treatment methods for lymphoproliferative diseases, such as lymphomas and lymphocytic leukemias, are lacking. Currently, conventional combination chemotherapy remains the principal treatment of choice for these disease states. The efficacy of chemotherapy is limited, especially when patients fail first line drug regimens. Other therapy modalities need to be developed to overcome the limitations of chemotherapy. Anti-idiotype antibodies directed against cell surface immunoglobulins of

individual lymphocytic neoplasms requires a lengthy and costly process to generate specific anti-idiotype antibodies for each patient individually, as each individual neoplasm likely has a unique idiotype. Moreover, development of anti-idiotype immunoglobulins depends primarily on immunization of nonhuman hosts, such as mice, which yields nonhuman immunoglobulins that are typically immunogenic when administered to humans. The necessity of humanization for therapeutic use of such antibodies would add to the cost and time expenditure, delaying treatment and making individualized anti-idiotype antibody therapy prohibitively expensive. Thus, although anti-idiotype antibodies theoretically are tools for diagnosis and therapy of B cell lymphomas, practical considerations hinder their widespread use in medicine.

Thus there exists a need in the art for methods and compositions for diagnosis and therapy of lymphoproliferative diseases and other diseases of abnormal cell proliferation, including lymphomas and lymphocytic leukemias. It is one object of the invention to provide methods and compositions for diagnosing and treating these pathological conditions, as well as treating immunological conditions which are amenable to targeted anti-proliferative therapy. The present invention fulfills these and other needs.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

SUMMARY OF THE INVENTION

In accordance with the foregoing objects, in one aspect of the invention are provided methods for identifying antiproliferative polypeptides which inhibit clonal expansion and/or induce apoptosis in cells of a predetermined cell population (e.g., a neoplastic cell sample) expressing a cell surface receptor which is a member of the immunoglobulin superfamily. A predetermined cell population expressing surface

immunoglobulin superfamily molecules is isolated from a patient as a cellular sample, such as a lymph node biopsy or blood sample or the like. The predetermined cell population or its clonal progeny propagated in culture are used as a source of specific surface immunoglobulin superfamily molecules for screening a polypeptide library, such as a bacteriophage peptide display library or a spatially defined polypeptide array on a solid substrate, to determine polypeptide sequences which bind the specific surface immunoglobulin superfamily molecules of the predetermined cell population with an affinity of about at least 1×10^5 M^{-1} or more (preferably at least 1×10^6 M^{-1} to 1×10^7 M^{-1}), and which substantially lack binding to irrelevant surface immunoglobulin superfamily molecules of other cells, thereby identifying candidate antiproliferative peptide sequences. Peptides and/or peptidomimetics comprising sequences corresponding to or substantially identical to the candidate antiproliferative peptide sequences are assayed, either in native form or crosslinked to additional candidate peptides or peptidomimetics, in cell culture. Such assays determine the biological activity(ies) of the peptide or peptidomimetic species by detecting at least one of the following functional properties: (1) binding to cell surface immunoglobulin superfamily molecules on the predetermined cell population and lacking substantial binding to irrelevant cell populations, (2) inhibition of cell proliferation of the predetermined cell population, (3) induction of apoptosis of the predetermined cell population, (4) stimulation of protein tyrosine kinase activity in the predetermined cell population treated with the peptide or peptidomimetic, (5) modulation of calcium flux across the plasma membrane, and (6) inhibition of other indicia of lymphocyte proliferation in the predetermined cell population but substantially lacking such inhibition in irrelevant cell populations. Biologically active antiproliferative peptides and peptidomimetics are then formulated for administration to a patient having a pathological condition that is characterized by abnormal proliferation of the predetermined cell population, such as a lymphoproliferative disease (e.g., a B cell lymphoma or

leukemia).

The invention also provides methods and compositions for identifying and administering anti-idiotype peptides and peptidomimetics for ablating or preventing clonal expansion of lymphocyte subpopulations expressing cell surface immunoglobulin superfamily molecules having specific idiotypes (i.e., antigen or ligand binding clefts). The anti-idiotype peptides and peptidomimetics are administered therapeutically or prophylactically to modulate immune system function by inactivating, by inducing clonal anergy or apoptosis, lymphocyte subpopulations which bind the anti-idiotype molecules.

In one aspect of the invention is provided diagnostic and therapeutic compositions of antiproliferative peptides and peptidomimetics which can be used in therapy and diagnosis of lymphoproliferative diseases in an individual patient. Diagnostic compositions can include imaging conjugates wherein anti-idiotype peptides or peptidomimetics are linked to imaging agents, such as by a covalent linkage or a noncovalent linkage which is substantially nonreleasable under physiological conditions (e.g., a streptavidin-biotin linkage). Therapeutic compositions comprise at least one species of antiproliferative peptide or peptidomimetic for treating lymphoproliferative diseases, other neoplastic conditions, or immune system disorders. Typical therapeutic compositions comprise an effective dosage of a anti-idiotype peptide or peptidomimetic in a pharmaceutically acceptable form, and optionally may include excipients or stabilizers.

The present invention provides compositions of peptides and peptidomimetics that have antiproliferative activity against lymphoma and lymphocytic leukemia cells, and more particularly against B cell lymphomas and B cell lymphocytic leukemias.

The present invention also provide methods for treating or preventing lymphoma and lymphocytic leukemia, and more particularly against B cell lymphomas and B cell lymphocytic leukemias in humans. A therapeutic method for treating a lymphoma or lymphocytic leukemia comprises delivering a therapeutically effective dosage of an antiproliferative peptide

to a patient having a lymphoma or lymphocytic leukemia expressing an idiotype which binds the antiproliferative peptide. The method may be used to treat other lymphoproliferative diseases as well. Antiproliferative peptides are non-immunoglobulin proteins and have advantageous pharmacological properties and are relatively inexpensive to identify and manufacture for a patient as compared to anti-idiotype immunoglobulins.

Also provided by the invention is a method for inhibition the proliferation of lymphoma cells and lymphocytic leukemia cells in culture. The method comprises delivering an antiproliferative dosage of an antiproliferative peptide to a cell culture comprising a lymphoma or lymphocytic leukemia expressing an idiotype which binds the antiproliferative peptide. Such methods will allow identification (and isolation) of variants of lymphoma and lymphocytic leukemia cells which have lost a proliferative phenotype and/or have lost expression of the immunoglobulin superfamily molecule to which the antiproliferative peptide has binding affinity for. Such variant cells may be used to screen compounds to identify antineoplastic pharmaceuticals and study the process of neoplastic variability.

Also provided in the invention is a method for inhibiting the proliferation of lymphoma cells or lymphocytic leukemia cells, comprising delivering an inhibitory dose of a non-immunoglobulin antiproliferative peptide which specifically binds to an immunoglobulin superfamily molecule idiotype present on the lymphoma cells or lymphocytic leukemia cells. Such a method can be used to treating a lymphoma or lymphocytic leukemia in a patient, comprising delivering a therapeutically effective dosage of a non-immunoglobulin antiproliferative peptide which specifically binds to an immunoglobulin superfamily molecule idiotype present on the lymphoma cells or lymphocytic leukemia cells.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows inhibition of anti-idiotype antibody binding to the relevant immunoglobulin with anti-idiotype peptide 3T802.

Fig. 2 shows the antiproliferative effect of biotinylated peptide 3T802 conjugated to streptavidin.

Fig. 3 shows a Western blot showing tyrosine phosphorylation in SupB8 (Tab) cells and the effect of peptide tetramers on signal transduction of SUP-B8 cells. (Panel A) Intracellular protein tyrosine phosphorylation in SUP-B8 cells stimulated with Peptide A tetramers: SUP-B8 cells were stimulated with medium (Lane 1), anti-IgM polyclonal serum (lane 2) and 5 μM control peptide tetramer (Lane 3) for two minutes, or with 5 μM Peptide A tetramer for 1, 2, 5, 10, or 15 minutes (Lanes 4-8). (Panel B) The blot was first stained with a goat anti-mouse IgG-Biotin antibody and detected with streptavidin HRP to show equal loading in all lanes of (Panel A). The experiment shown is representative of 4 experiments with similar results.

Fig. 4 shows binding of SUP-B8 Ig and control immunoglobulins to biotinylated peptides. Biotinylated peptide monomers were immobilized on a streptavidin coated microtiter plate. The binding of purified SUP-B8 Ig, purified Ig from other patients' tumors, purified Ig from the lymphoma cell line SU-DHL4 or purified polyclonal normal IgM was measured in an ELISA. The isotype of each immunoglobulin is shown in the legend (λ:lambda, κ:kappa). Data shown is the mean of duplicate samples +/- SD. The data is representative of three experiments with similar results.

Fig. 5 shows inhibition of anti-Id antibody binding to SUP-B8 Ig with peptides. Peptide monomers were added at increasing concentrations to inhibit the binding of the anti-id antibody to SUP-B8 Ig, measured in an ELISA assay. • Peptide A, ■ Peptide B, ▲ Peptide C, O Control Peptide D. Data shown is the mean of duplicate samples +/- SD. The data is representative of five experiments with similar results.

Fig. 6 shows the effect of peptide monomers and tetramers on cell proliferation measured by ^{3}H-thymidine

incorporation. Panel A: SUP-B8 cells were incubated with peptide monomers or streptavidin at varying concentrations for 72 hours. The streptavidin concentrations used were 1/4 of those shown, to be identical to the concentration of streptavidin used for Panels B and C. • Peptide A, ■ Peptide B, ▲ Peptide C, ♦ Streptavidin. SUP-B8 cells (Panel B) or OCI-Ly8 Cells (Panel C) were incubated with peptide tetramers at varying concentrations for 72 hours • Peptide A tetramer, ■ Peptide B tetramer, ▲ Peptide C tetramer, o Control Peptide D tetramer. Data shown are means of raw counts per minute of quadruplicate samples +/- SD. The experiments shown are representative of 3, 8 and 3 experiments with similar results for (Panel A), (Panel B) and (Panel C) respectively.

Fig. 7 shows the effect of peptide dimers on cell proliferation measured by ^{3}H-thymidine incorporation, (panel A) A variant of peptide A (KPWWVSRVSSC) (•) and a control peptide (YSSVSRVWWPK) (o) which includes the reverse octapeptide sequence of peptide A, were subjected to a dimerization procedure and incubated for 72 hours with SUP-B8 cells. SUP-B8 cells (Panel B) or OCI-Ly8 Cells (Panel C) were incubated with tandem repeat peptides at varying concentrations for 72 hours. ▲ Tandem Repeat Peptide C, ▵ Tandem Repeat Scrambled Peptide C. Data shown are means of raw counts per minute of triplicate (Panel A) or quadruplicate (panel B), (Panel C) samples +/- SD. The experiments shown are representative of 3 experiments with similar results.

Fig. 8 shows DNA fragmentation in SUP-B8 cells incubated with peptide tetramers. DNA from SUP-B8 cells incubated for 24 hours with 2.5 μM peptide tetramers was electrophoresed on an agarose gel and stained with ethidium bromide. Lane 1: Peptide A tetramer, lane 2: Peptide B tetramer, lane 3: Peptide C tetramer, lane 4: Scrambled Peptide C tetramer, lane 5: Reverse Peptide C tetramer. The experiment shown is representative of 5 experiments with similar results.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood

by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage (Immunology - A Synthesis, 2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Massachusetts (1991), which is incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ϵ-N,N,N-trimethyllysine, ϵ-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline).

Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

As used herein, the term "antiproliferative peptide"

refers to a polypeptide that inhibits proliferation or induces apoptosis in a predetermined cell population that expresses a cell surface immunoglobulin superfamily molecule having an idiotype which is substantially absent in other cell populations bearing cell surface immunoglobulin superfamily molecules. Antiproliferative peptides typically have structures and chemical properties defining an epitope which binds to an idiotype-determining binding cleft of an immunoglobulin superfamily molecule, such as a surface IgM or IgD molecule. Antiproliferative peptides are typically polypeptides, although they may comprise D- amino acids in peptide linkage. Alternatively, antiproliferative peptidomimetics comprise polypeptide-like polymers that contain novel backbone structures or unnatural amino acids (Ellman et al. (1992) Science 255: 197, which is incorporated herein by reference), or other non-peptide chemical constituents, including peptoids (Simon et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 9367. Antiproliferative peptides and peptidomimetics generally comprise epitopes which are bound at an idiotype-determining binding cleft (e.g., antigen binding site of an immunoglobulin) and are generally anti-idiotype peptides and peptidomimetics. However, some antiproliferative peptides and peptidomimetics may induce apoptosis, clonal anergy, or proliferative arrest by a mechanism other than binding to an idiotype-determining binding portion of an immunoglobulin superfamily molecule; such antiproliferative peptides may possess the property of inducing apoptosis, clonal anergy, or proliferative arrest in cells bearing immunoglobulin superfamily molecules of various idiotypes.

The term "antineoplastic peptide" is used herein to refer to peptides that have the functional property of inhibiting development or progression of a neoplasm in a human, particularly a lymphoma, lymphocytic leukemia, or pre-leukemic condition.

The term "candidate antineoplastic peptide" is used herein to refer to a peptide which is identified by one or more screening method(s) of the invention as a putative antineoplastic peptide. Some candidate antineoplastic peptides may have therapeutic potential as anticancer drugs for human use.

As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g., ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{125}I, ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

As used herein "normal blood" or "normal human blood" refers to blood from a healthy human individual who does not have an active neoplastic disease or other disorder of lymphocytic proliferation, or an identified predisposition for developing a neoplastic disease. Similarly, "normal cells", "normal cellular

sample", "normal tissue", and "normal lymph node" refers to the respective sample obtained from a healthy human individual who does not have an active neoplastic disease or other lymphoproliferative disorder.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence.

The terms "substantial similarity" or "substantial identity" as used herein denotes a characteristic of a polypeptide sequence or polynucleotide sequence, wherein the polypeptide sequence has at least 50 percent sequence identity compared to a reference sequence, and the nucleic acid sequence has at least 70 percent sequence identity compared to a reference sequence. The percentage of sequence identity is calculated excluding small deletions or additions which total less than 25 percent of the reference sequence. The reference sequence may be a subset of a larger sequence, such as a polypeptide epitope of a larger polypeptide sequence; however, the reference sequence is at least 6 amino residues long in the case of a polypeptide.

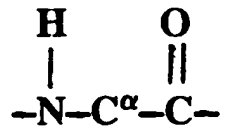
As used herein, the terms "biological activity" and "antiproliferative activity" are defined as the capacity to inhibit specifically the proliferation, clonal expansion, or immunologic activation (e.g., production of secreted immunoglobulins) of a predetermined cell population. Thus, an antiproliferative peptide of the invention has biological activity if a predetermined cell population that has been treated with a suitable concentration of the antiproliferative peptide produces a detectable decrease in cell proliferation (e.g., ^{3}H-thymidine incorporation, cell number) as compared to a parallel cell culture that is not treated with the antiproliferative peptide and as compared to a cell culture of irrelevant cells (i.e., cells not bearing immunoglobulin superfamily molecules of the same idiotype as the predetermined cell population). Suitable concentrations (i.e., efficacious dose) of antiproliferative peptides and peptidomimetics can be determined

by various methods, including generating an empirical dose-response curve, predicting potency and efficacy of a congener by using QSAR methods or molecular modeling, and other methods used in the pharmaceutical sciences (see Fauchere, J. (1986) Adv. Drug Res. 15: 29, which is incorporated herein by reference). Antiproliferative peptides of the invention also have specificity for inhibiting proliferation of the predetermined cell population. Thus, a biologically active peptide of the invention specifically inhibits proliferation of one or more predetermined cell populations without producing significant nonspecific cytotoxicity in irrelevant cells. Significant nonspecific cytotoxicity can be determined by various methods, including, for example, determining that treatment of an irrelevant cell culture with an efficacious dose of an agent reduces cell proliferation by at least about 50 percent as compared to a parallel culture that is not treated with the peptide. Other type of assays that can be used to ascertain nonspecific cytotoxicity in cell cultures include: Trypan Blue dye exclusion, incorporation of radiolabeled amino acids and/or radiolabeled nucleotides, mitotic rate (e.g., doubling time), and other methods known in the art.

Polypeptide Nomenclature and Structure Convention

In describing the invention, both single-letter and three-letter abbreviations of the 20 conventional amino acids are used.

The peptide backbone of a polypeptide consists of a repeated sequence of three atoms: the amide N, the α C, and the carbonyl C:



which are generally represented as N_i, C_i^{α}, and C_i', respectively, where i is the number of the residue, starting from the amino end. In the polypeptide notation used herein, the

lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention.

An open hyphen at the lefthand end of a peptide sequence indicates that an amino-terminal peptide extension (including unconventional amino acids) may be attached to the amide nitrogen of the leftmost amino acid residue in the given sequence. If there is no open hyphen at the lefthand end of a peptide sequence, that indicates that the leftmost residue is the amino-terminus of the peptide, although the amide nitrogen of the leftmost residue may be chemically modified, for example with an acetyl or methyl group, but typically the amide nitrogen is bonded to the α carbon and hydrogen substituents only.

An open hyphen at the righthand end of a peptide sequence indicates that a carboxy-terminal peptide extension (including unconventional amino acids) may be attached to the carbonyl carbon of the rightmost residue in the given sequence. If there is no open hyphen at the righthand end of a peptide sequence, that indicates that the rightmost residue is the carboxy-terminus of the peptide, although the carbonyl carbon of the rightmost residue may be chemically modified, for example by amidation, but typically the carbonyl carbon is bonded to a hydroxyl group, forming a carboxyl group attached to the α carbon of the rightmost residue.

For example, the sequence -Lys-Pro-Trp- and the carboxy-terminal sequence -Ser-Arg-Val both occur in the octapeptide Lys-Pro-Trp-Tyr-Val-Ser-Arg-Val. However, Lys-Pro-Trp designates a tripeptide since there are no righthand or lefthand open hyphens.

DETAILED DESCRIPTION

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, polypeptide synthesis, generation and

propagation of bacteriophage peptide display libraries and the like and microbial culture and transformation (e.g., electroporation). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) CSH Press, Cold Spring Harbor, N.Y.; and Antibodies: A Laboratory Manual, (1988) E. Harlow and D. Lane, CSH Press, Cold Spring Harbor, NY, and Goodspeed et al. (1989) Gene 76: 1; Dunn et al. (1989) J. Biol. Chem. 264: 13057) which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

The recognized immunoglobulin superfamily is described in The Immunoglobulin Gene Superfamily, A.F. Williams and A.N. Barclay, in Immunoglobulin Genes, T. Honjo, F.W. Alt, and T.H. Rabbitts, eds., (1989) Academic Press: San Diego, CA, pp.361-387, which is incorporated herein by reference.

Although the methods presented below are described specifically for identifying antiproliferative peptides which bind to immunoglobulin superfamily molecules, it is believed that the method is generally applicable for identifying antiproliferative peptides which bind to non-immunoglobulin cell surface receptors, particularly those receptors which mediate tyrosine kinase activity which is modulatable upon ligand binding.

General Methods

A basis of the present invention is the unexpected finding that peptides having defined sequences can inhibit proliferation, induce clonal anergy, mediate a signal that activates the cell through a tyrosine kinase regulated cell proliferation pathway, and/or induce apoptosis in cells, such as lymphocytic cells of a lymphoma or lymphocytic leukemia, by

binding to cell surface immunoglobulin superfamily proteins having specific idiotypes which bind the peptides. Most neoplasms represent a clonal expansion of a single transformed cell, and thus, for example, lymphocytes of any B cell lymphoma generally express a single species of surface immunoglobulin having an idiotype characteristic of that individual B cell lymphoma. For a given individual lymphoma or lymphocytic leukemia it is generally possible to define, by screening libraries of peptides representing a variety of peptide sequences, specific polypeptide sequences which bind to the cell surface immunoglobulin superfamily molecules (e.g., surface IgM or IgD) having the idiotype characteristic of that individual B cell lymphoma. The peptides identified by such screening methods are tested for the ability to inhibit proliferation, induce clonal anergy, modulate tyrosine kinase activity, and/or induce apoptosis in cultured cells of the individual B cell lymphoma, either as individual peptides (optionally including repeat anti-idiotype motifs) or as complexes of crosslinked peptides. Peptides which inhibit proliferation, induce clonal anergy, modulate tyrosine kinase activity, and/or induce apoptosis in the cultured lymphoma cells are thereby identified as antiproliferative (or anti-idiotype) peptides and may be administered to a patient which has a B cell lymphoma expressing immunoglobulin superfamily molecules with the characteristic idiotype. The tissue penetration of peptides, the ease of synthesis and the ability to modify peptides is superior to anti-idiotype antibodies. Moreover, the peptides of the present invention tend to be less immunogenic than non-human monoclonal antibodies. Thus the Ig receptor can be targeted with peptides that bind specifically to the individual Ig receptor species as surrogate ligands. The peptides themselves, when made multimeric, inhibit growth by crosslinking Ig receptors, and/or can be conjugated to deliver toxins or radionuclides to neoplastic cells bearing the Ig receptor species to which the peptide(s) bind.

It also has been found that such anti-idiotype peptide screening methods can define multiple peptide sequences which

bind to the same immunoglobulin idiotype. By comparison of the multiple anti-idiotype peptide sequences, at least one consensus anti-idiotype peptide sequence motif can be generated which can be used as a sequence template for the generation of additional peptide sequence variants having binding affinity for the immunoglobulin superfamily molecule. Typically, such peptide sequence variants comprise one or two amino acid variations from the anti-idiotype peptide sequence motif, frequently three amino acid variations, and occasionally four or more amino acid variations from the anti-idiotype peptide sequence motif (which may itself comprise alternative residues at specific amino acid positions), and may comprise additional amino acid sequences in polypeptide linkage to the portion containing the consensus motif, either amino-terminal extensions, carboxyterminal extensions, or both. The anti-idiotype peptide consensus motif constitutes a convenient description of the structural and conformational characteristics of peptides which bind to the idiotypic binding site (e.g., the antigen binding site of an immunoglobulin).

In addition to anti-idiotype peptides, a consensus motif may form the basis for synthesis of peptidomimetics. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) Adv. Drug Res. 15: 29; Veber and Freidinger (1985) TINS p.392; and Evans et al. (1987) J. Med. Chem 30: 1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: $-CH_2NH-$, $-CH_2S-$, $-CH_2-CH_2-$, $-CH=CH-$ (cis and trans), $-COCH_2-$, $-CH(OH)CH_2-$, and $-CH_2SO-$, by methods known in

the art and further described in the following references: Spatola, A.F. in "Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J.S., Trends Pharm Sci (1980) pp. 463-468 (general review); Hudson, D. et al., Int J Pept Prot Res (1979) 14:177-185 ($-CH_2NH-$, CH_2CH_2-); Spatola, A.F. et al., Life Sci (1986) 38:1243-1249 ($-CH_2-S$); Hann, M.M., J Chem Soc Perkin Trans I (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G. et al., J Med Chem (1980) 23:1392-1398 ($-COCH_2-$); Jennings-White, C. et al., Tetrahedron Lett (1982) 23:2533 ($-COCH_2-$); Szelke, M. et al., European Appln. EP 45665 (1982) CA: 97:39405 (1982) ($-CH(OH)CH_2-$); Holladay, M.W. et al., Tetrahedron Lett (1983) 24:4401-4404 ($-C(OH)CH_2-$); and Hruby, V.J., Life Sci (1982) 31:189-199 ($-CH_2-S-$); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is $-CH_2NH-$. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (e.g., immunoglobulin superfamily molecules) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (e.g., labelling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g.,

D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch (1992) Ann. Rev. Biochem. 61: 387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

Crosslinking of Anti-Idiotype Peptides

It has also been unexpectedly found that crosslinking anti-idiotype peptides, either by linking multiple molecules of a single species of anti-idiotype peptide or by linking multiple species of anti-idiotype peptides, results in enhanced biological activity for inhibiting proliferation, inducing clonal anergy, and/or inducing apoptosis in cells expressing cell surface immunoglobulin superfamily molecules of the characteristic idiotype used for screening the peptide library. Crosslinking is typically accomplished by synthesizing biotinylated anti-idiotype peptides and contacting them with streptavidin under aqueous binding conditions (e.g., buffered physiological saline, optionally including Tween and/or nonspecific blocking protein) to form crosslinked anti-idiotype peptides. Alternatively, anti-idiotype peptides may be crosslinked with a covalent crosslinking agent, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce Chemical Co.) or succinimidyl 3-(2-pyridyldithio)propionate (Pierce Chemical Co.) according to methods described in the art (Gilliland et al. (1980) Proc. Natl. Acad. Sci. (U.S.A.) 77: 4539; Wu and Wu (1987) J. Biol. Chem. 262: 4429; Wu and Wu (1988) J. Biol. Chem. 263: 14621; Wu and Wu (1988) Biochemistry 27: 887; Wu et al. (1989) J. Biol. Chem. 264: 16985; Cotten et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 4033; Wagner et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 3410; Zenke et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 3655; and Wagner et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4255, incorporated herein by reference. Peptides can be crosslinked through carboxy-terminal cysteines (e.g., which may

be added carboxyterminal to a consensus sequence or substantially identical variant), typically using spacers such as bis-maleimidohexane (BMH) (Pierce Chemical Co.) which can covalently link two sulfhydryl groups. Preferably, crosslinking occurs at non-interfering positions so that substantial biological activity (e.g., antiproliferative activity) is retained. Such non-interfering positions generally are positions that do not form direct contacts with the immunoglobulin superfamily molecule(s) (e.g., surface IgM) to which the peptide or peptidomimetic binds to produce the therapeutic effect.

Alternatively, or in combination with chemical crosslinking, polypeptides consisting essentially of repeats of a peptide consensus sequence (or substantially identical variants thereof) may be linked in peptidyl linkage, typically including a spacer between the repeats comprising a noninterfering amino acid, such as glycine. Such repeat polypeptides can function as a crosslinked complex in inhibiting proliferation, inducing clonal anergy, mediating a signal that activates the cell through a tyrosine kinase regulated cell proliferation pathway, and/or inducing apoptosis in cells.

Generally, the crosslinked peptides will form dimers and/or tetramers or higher order polymers for advantageous therapeutic efficacy. In addition to their use for therapy of neoplastic disease in vivo and/or ex vivo (e.g., to purge explanted hematopoietic and/or lymphocytic cells of undesired neoplastic cells expressing the relevant immunoglobulin superfamily idiotype), such antiproliferative peptides can be used in vitro and in vivo as commercial research reagents. For example and not limitation, antiproliferative peptides to a transplantable human lymphoma model (e.g., SUP-B8 lymphoma line in SCID mice or in culture) can be sold to researchers for standardizing assays (e.g., screening assays for identifying novel agents having antineoplastic/antiproliferative activity), for manipulating the proliferative status of the cell line, and/or for inducing a reproducible model of apoptosis, and the like, among many other uses which make the antiproliferative peptides commercially marketable to the research community (e.g.,

similar to restriction enzymes, Taq polymerase, and the like). Antiproliferative peptides to essentially any lymphocytic cell line expressing a surface immunoglobulin superfamily signal transduction molecule can be produced by the methods of the invention; thus antiproliferative peptides can be generated as custom research reagents (e.g., for commercial sale) for essentially any lymphocytic cell line (i.e., in addition to SUP-B8) used in research and diagnostics.

In particular, the antiproliferative peptides of the invention may be sold in kit form for research and diagnostic usage, either with or without a sample of the cell line(s) which were used to identify the antiproliferative peptides and/or which are induced to undergo apoptosis upon treatment with an efficacious dose of the antiproliferative peptides. In some embodiments, the kits include vials or ampules of such viable cells which are further capable of propagation in a host animal (e.g., immunodeficient or immunocompromised mouse; SCID mouse) as a model of a lymphoproliferative disease or other hematopoietic disorder (e.g., neoplasia); and/or for providing a mechanistic model of lymphocyte apoptosis in vivo, and the like.

Identifying Anti-Idiotype Peptides

A predetermined cell population is a population of cells which is substantially comprised of cells that are the clonal progeny of a precursor cell (e.g., stem cell, neoplastically transformed cell) and which express a surface immunoglobulin superfamily molecule having an identical idiotype. For example but not limitation, a biopsy sample of a B cell lymphoma comprises a predetermined cell population since a substantial fraction of the cells in the biopsy sample are derived from a common lymphopoietic precursor cell (i.e., presumably the transformed parent cell) and also express a surface immunoglobulin having essentially identical structure and antigen binding properties (i.e., identical idiotype). It is recognized that some degree of somatic mutation may occur so that minor sequence variations in the immunoglobulin may occur yet not

alter idiotypic specificity.

A predetermined cell population comprising cells which express a cell surface immunoglobulin superfamily molecule, such as a T cell receptor (α/β) or a surface IgM or IgD, is obtained, typically by obtaining a blood sample enriched for a clone of lymphoid cells or lymph node biopsy from a patient suspected of having a lymphoproliferative disease, such as a lymphoma or lymphocytic leukemia (ALL or CLL). The explanted lymphocytic cells may be cultured in a suitable culture medium under growth conditions, may be used directly, or may be used to generate a hybridoma by fusion with an immortalized lymphoid cell line. If the explanted cells are B cells, it is generally preferable to obtain secreted immunoglobulin produced by the cells of hybridomas formed by fusion of the cells with a suitable fusion partner. Alternatively, the predetermined cell population may be used directly for screening. Alternatively, the immunoglobulin superfamily molecule(s) can be recovered from cell lysates or extracts made from the cells, generally via immunoaffinity purification using antibodies directed against the immunoglobulin superfamily molecules (e.g., with an anti-human IgM antibody). For recovery of membrane-bound immunoglobulin superfamily molecules, a mild detergent, such as deoxycholate, may be included to solubilize the molecules.

Immunoglobulin produced by the predetermined cell population (or hybridomas derived therefrom) may be used to screen a peptide library for identifying peptides which bind specifically to the immunoglobulin on the basis of idiotypic identity. Alternatively, the cells of the predetermined cell population may be used to screen a peptide library directly, thereby identifying peptide species which bind to the immunoglobulin superfamily molecule species expressed on the surface of the cells as candidate anti-proliferative peptides.

Various embodiments of peptide libraries may be screened to identify anti-idiotype peptides. Two preferred peptide library types are: (1) a bacteriophage peptide display library, which can comprise a bacteriophage antibody display library for selecting anti-idiotype antibodies, and (2) a

spatially defined array of peptides fixed to a solid support (U.S. Patent 5,143,854, incorporated herein by reference). Alternative peptide libraries suitable for use have been described in the art, including U.S.S.N. 07/963,321; U.S.S.N. 07/778,233; and Cull et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89:1865, incorporated herein by reference.

Various bacteriophage peptide display libraries are suitable for screening to identify anti-idiotype peptide sequences, including but not limited to those described in PCT/US91/04384; U.S.S.N. 07/718,577; U.S.S.N. 07/517,659; Cwirla et al. (1990) Proc. Natl. Acad. Sci. (U.S.A.) 87: 6378; de la Cruz et al. (1988) J. Biol. Chem. 263: 4318; Parmley and Smith, Gene 73:305-318 (1988); Scott and Smith (1990) Science 249: 386; Devlin et al. (1990) Science 249: 404, incorporated herein by reference. Such libraries are screened by a variety of methods, preferably by selective affinity adsorption to a predetermined antigen (e.g., a relevant immunoglobulin or cell of the predetermined cell population). Such libraries can be used to find ligands or surrogate ligands to receptors.

Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 4363; Clackson et al. (1991) Nature 352: 624; McCafferty et al. (1990) Nature 348: 552; Burton et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 10134; Hoogenboom et al. (1991) Nucleic Acids Res. 19: 4133; Chang et al. (1991) J. Immunol. 147: 3610; Breitling et al. (1991) Gene 104: 147; Marks et al. (1991) J. Mol. Biol. 222: 581; Barbas et al. (1992) Proc. Natl. Acad. Sci. (U.S.A.) 89: 4457; Hawkins and Winter (1992) J. Immunol. 22: 867; Marks et al. (1992) Biotechnology 10: 779; Marks et al. (1992) J. Biol. Chem. 267: 16007; Lowman et al (1991) Biochemistry 30: 10832; Lerner et al. (1992) Science 258: 1313, incorporated herein by reference). Such antibody display libraries may be used to rapidly identify anti-idiotype antibodies by the methods of the invention.

One important method involves the display of a biological molecule, such as a peptide, antibody, or other

protein (collectively referred to as "(poly)peptide") on the surface of a phage or cell. These methods typically involve establishing a physical or logical connection between each (poly)peptide and the nucleic acid that encodes the (poly)peptide; perhaps the best known method in this category involves the presentation of a (poly)peptide on the surface of a filamentous phage. The phage can be incubated with an immobilized receptor (e.g., a relevant immunoglobulin) of interest, so that phage which present a (poly)peptide that binds to the receptor can be separated from phage that do not. After several rounds of affinity enrichment and phage replication, followed by isolation of the phage that bind and sequence determination of the phage nucleic acid, this method allows one to identify the sequence of (poly)peptide ligands for the receptor. Such methods are described in more detail in PCT patent publication Nos. 91/17271; 91/18980, and 91/19818.

Another important recombinant method for the display of (poly)peptide ligands involves the production of a fusion protein composed of a protein that specifically binds to DNA and the potential (poly)peptide ligand. In one embodiment of this method, the library of (poly)peptides is produced by transforming recombinant host cells with a vector that encodes a lac repressor/(poly)peptide fusion protein and contains a lac operator sequence. When the transformed host cells are cultured under conditions that allow for expression of the fusion protein, the fusion protein binds to the vector that encodes the fusion protein. Upon lysis of the host cells, the fusion protein/vector complexes can be screened against a receptor in much the same way the phage are screened in the phage-based display method.

In contrast to the recombinant methods, in vitro chemical synthesis provides a method for generating libraries of compounds, without the use of living organisms, that can be screened for ability to bind to a receptor. Although in vitro methods have been used for quite some time in the pharmaceutical industry to identify potential drugs, recently developed methods have focused on rapidly and efficiently generating and screening large numbers of compounds. One early method involves the

synthesis of peptides on a set of pins or rods. See PCT patent publication Nos. 84/03506 and 84/03564. Another method involves the use of a synthesis resin or beads and a variety of flow-through containers into which the beads are placed. The containers are then exposed to monomer-coupling solutions and labeled to indicate the monomer coupling reactions to which the container has been exposed (see, U.S. Patent No. 4,631,211). A related method dispenses with the labeling step and separate containers for each peptide to achieve greater diversity at the cost of easy identification of a particular ligand of interest. In this method, the synthesis beads are pooled and redistributed after each set of monomer coupling reactions. After screening with a receptor, the ligands on a bead of interest must be identified by removing the ligand from the bead and determining the molecular structure of the ligand (see, PCT patent publication No. 92/00091).

A significant improvement over this latter method involves tagging each bead with an identifier tag, such as an oligonucleotide, so as to facilitate ligand identification. Another powerful method for generating large collections of compounds addresses the ligand identification problem by forming arrays of different compounds in a manner that places each different compound of the array at a discrete, predefined location. The location identifies each ligand. This method, called very large scale immobilized polymer synthesis, is described in U.S. patent No. 5,143,854; PCT patent publication Nos. 90/15070 and 92/10092; Fodor et al. (1991) Science 251: 767; and Dower and Fodor (1991) Ann. Rep. Med. Chem. 26: 271.

Other systems for generating libraries of compounds have aspects of both the recombinant and in vitro chemical synthesis methods. In these hybrid methods, biological enzymes or enzyme complexes play an important role in generating the compounds, but no living organisms or cells are directly used. The technique of in vitro translation has been used to synthesize proteins of interest (see PCT patent publication Nos. 88/08453, 90/05785, 90/07003, and 91/02076); this technique has also been proposed as a method to generate large libraries of peptides

(see, PCT patent publication Nos. 91/05058 and 92/02536).

Typically, the peptide library is screened by contacting the library with a predetermined cell population (e.g., lymphoma cells) or the immunoglobulin superfamily molecule from the predetermined cell population under aqueous binding conditions and identifying peptides which bind the cells or immunoglobulin from the predetermined cell type but which substantially do not bind irrelevant cells or immunoglobulin derived from irrelevant cells. Such peptides are identified as candidate antiproliferative peptides which may be further evaluated for antiproliferative activity by in vitro assays using cultured cells of the predetermined cell population or freshly obtained cells (e.g., can comprise thawed aliquots) from a biopsy specimen.

For example and not limitation, the following description of a method to identify anti-idiotypic peptides which bind to an immunoglobulin idiotype characteristic of a predetermined cell population by screening a bacteriophage peptide display library is provided. A bacteriophage peptide display library displaying peptide sequences from 6 to 20 amino acids in length (typically as a fusion with a phage coat protein such as pIII of a filamentous bacteriophage) is optionally precleared for non-idiotype-specific binding by contacting the library of bacteriophage particles with an irrelevant class-matched immunoglobulin (e.g., a human serum Ig fraction obtained from an individual other than the patient) bound to Sepharose under aqueous binding conditions, and bacteriophage particles bound to the immobilized irrelevant immunoglobulin are removed by separation of the solid phase (e.g., Sepharose). The remaining precleared library is panned over (i.e, contacted with in aqueous binding conditions) irrelevant immunoglobulin coupled to a solid phase (e.g., a plastic Petri plate) and bacteriophage immobilized by binding to the irrelevant immunoglobulin are removed and discarded with the solid phase. The remaining bacteriophage in the supernatant are panned over relevant immunoglobulin (i.e., immunoglobulin derived from the predetermined cell population) immobilized on a solid support

and/or immobilized cells of the predetermined cell population (if cells are used, it is preferred that the library is precleared against an equivalent cell type, e.g., a B lymphocyte population). Alternatively, preclearance of the bacteriophage peptide display library may be omitted and the phage population screened directly by panning.

Bacteriophage which do not bind to the immobilized relevant immunoglobulin or immobilized cells remain unbound in the supernatant and are discarded, whereas bacteriophage which are immobilized by specific binding to the relevant immunoglobulin remain bound and are separated from the supernatant by removal of the supernatant and, optionally, rinsing the immobilized fraction with a mild solution (e.g., PBS). Phage displaying peptides without the desired specificity are removed by washing. The degree and stringency of washing required will be determined for each relevant immunoglobulin of interest. A certain degree of control can be exerted over the binding characteristics of the anti-idiotype peptides recovered by adjusting the conditions of the binding incubation and the subsequent washing. The temperature, pH, ionic strength, divalent cations concentration, and the volume and duration of the washing will select for anti-idiotype peptides within particular ranges of affinity for the relevant immunoglobulin. Selection based on slow dissociation rate, which is usually predictive of high affinity, is a practical route. This may be done either by continued incubation in the presence of a saturating amount of a known anti-idiotypic peptide or antibody for the relevant immunoglobulin idiotype, or by increasing the volume, number, and length of the washes. In each case, the rebinding of dissociated peptide-displaying phage is prevented, and with increasing time, peptide-displaying phage of higher and higher affinity are recovered.

Bound bacteriophage are recovered from the immobilized fraction by elution with a known anti-idiotype peptide ligand or with an acidic solution (e.g., pH 2.0 to 2.5), adjusted to neutral pH, and propagated in bacterial hosts to amplify the library of recovered bacteriophage. The recombinant

bacteriophage display vectors comprising the displayed peptide which bound to the relevant immunoglobulin are transformed into bacterial cells (e.g. E. coli) wherein they are expressed and phage particles are assembled to form an enriched bacteriophage antibody phage display library.

Such enriched libraries typically are screened further by at least one additional cycle of affinity selection as described above with immobilized relevant immunoglobulin and comprising a low pH (e.g., pH 2.1) elution step. Typically, at least about 2 to 5 cycles of this enrichment procedure (optionally including additional subtractive panning over irrelevant immunoglobulin or cells) are performed and specific anti-idiotype binding clones are isolated.

Once isolated, DNA from the anti-idiotype binding clones is usually prepared and the displayed peptide region sequences are isolated and the nucleotide sequence is determined by sequencing and/or is ligated into a suitable expression vector for high-level expression in bacterial or eukaryotic host cells. The nucleotide sequence of a displayed peptide from a selected clone encodes a putative anti-idiotype peptide. The anti-idiotype peptides thus identified may be used for human therapeutics (e.g., as antiproliferative peptides or toxin conjugates), medical diagnostics (e.g., in vivo imaging of peptide-conjugated imaging agents) and in vitro diagnostic assays (e.g., immunohistochemical staining of histopathological specimens to track recurrence or metastatic disease in a patient).

A variety of techniques can be used in the present invention to diversify a peptide library or to diversify around anti-idiotype peptides found in early rounds of panning to have sufficient binding activity. In one approach, the positive phage (those identified in an early round of panning) are sequenced to determine the identity of the active peptides. Oligonucleotides are then synthesized based on these peptide sequences, employing a low level of all bases incorporated at each step to produce slight variations of the primary oligonucleotide sequences. This mixture of (slightly) degenerate oligonucleotides is then cloned

into the affinity phage as described herein. This method produces systematic, controlled variations of the starting peptide sequences. It requires, however, that individual positive phage be sequenced before mutagenesis, and thus is useful for expanding the diversity of small numbers of recovered phage and selecting variants having higher binding affinity and/or higher binding specificity.

Alternatively, or in combination with peptide display libraries (e.g., bacteriophage display libraries), other peptide screening methods can be used to select antiproliferative (anti-idiotype) peptides that bind to the relevant immunoglobulin superfamily molecule. For example, solid phase spatially-defined peptide arrays (VLSIPS; U.S. Patent No. 5,143,854), polysome nascent peptide libraries, yeast two-hybrid systems (Chien et al. (1991) Proc. Natl. Acad. Sci. (U.S.A.) 88: 9578; Zervos et al. (1993) Cell 72: 223), and other systems (e.g., (Germino et al. (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 933; Guarente L (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 1639) and the like which can be used for selecting peptide sequences which bind to a predetermined polypeptide (e.g., Ig superfamily molecule idiotype) can be used to identify anti-idiotype peptide sequences.

Frequently, the screening protocol can comprise a yeast two-hybrid system wherein the yeast cells express: (1) a first fusion protein comprising the relevant immunoglobulin superfamily molecule variable region domain (e.g., Ig variable region domains) and a first transcriptional regulatory protein sequence (e.g., GAL4 activation domain), (2) a second fusion protein comprising a random, pseudorandom, or defined sequence peptide sequence and a second transcriptional regulatory protein sequence (e.g., GAL4 DNA-binding domain), and (3) a reporter gene (e.g., β-galactosidase) which is transcribed when an intermolecular complex comprising the first fusion protein and the second fusion protein is formed. Yeast cells that express the reporter gene are selected (e.g., on the basis of β-gal expression and/or growth) and the random, pseudorandom, or defined sequence peptide sequence of the second fusion protein(s) are determined (e.g.,

by DNA sequencing). Peptides comprising (or consisting essentially of) the selected random, pseudorandom, or defined sequence peptide sequence(s) are candidate antiproliferative peptides. Anti-idiotype peptides can be used to bind specifically to lymphocytes expressing the relevant immunoglobulin superfamily molecule idiotype, such as to target linked toxins or radionuclides to such cells, or (if labeled or otherwise detectable) to identify such cells in a cellular sample (e.g, a blood sample from a patient undergoing chemotherapy to monitor therapeutic progress and/or recurrence) regardless of whether the anti-idiotype peptide has antiproliferative effects.

Identifying Antiproliferative Peptides

The anti-idiotype peptides identified by screening of a peptide library are thereby identified as candidate antiproliferative peptides. To establish that a candidate antiproliferative peptide possesses antiproliferative activity, the peptide (usually in dimer or higher multimeric form by crosslinking with a linker) is administered to a cell culture containing the predetermined cell population in growth conditions (e.g., RPMI with fetal bovine serum) and the peptide's activity in producing one or more of the following: (1) inhibition of cell proliferation of the predetermined cell population, (2) induction of apoptosis of the predetermined cell population, (3) stimulation of protein tyrosine kinase activity in the predetermined cell population treated with the peptide or peptidomimetic, (4) modulation of calcium flux across the plasma membrane, and (5) inhibition of other indicia of lymphocyte proliferation (e.g., ^{3}H-thymidine incorporation) in the predetermined cell population but substantially lacking such inhibition in irrelevant cell populations.

Peptides identified as antiproliferative peptides may be further evaluated by their capacity to reduce tumorigenesis or neoplastic cell burden in nu/nu or SCID mice harboring a transplant of the predetermined cell population, as compared to untreated mice harboring an equivalent transplant of the predetermined cell population.

Generally, antiproliferative peptides are at least about 4 amino acids in length, typically at least 5 or 6 amino acids in length, often at least seven or eight amino acids long, and frequently 10 to twelve amino acids long or more, and such peptides are often dimerized. Minimal anti-idiotypic and antiproliferative peptides are often less than 13 amino acids in length, and frequently less than 9 amino acids in length. A frequent size range is from 8 to 12 amino acids, with preferred sizes depending upon the specific idiotypic binding characteristics of the subject relevant immunoglobulin superfamily protein.

Antiproliferative peptides and peptidomimetics which possess antiproliferative activity towards the predetermined cell population may be formulated for therapeutic and diagnostic administration to a patient having a lymphoproliferative disease (e.g., a B cell lymphoma) comprising a clonal expansion of cells having surface immunoglobulin with substantially the same idiotype as the predetermined cell population. Typically, the antiproliferative peptides and peptidomimetics are administered to the patient from whom the predetermined cell population was obtained.

Some antiproliferative peptides may comprise N-terminal and C-terminal additions, generally comprising one or more amino acids in peptide linkage, although non-peptide linkage chemistries (e.g., esterification) and/or chemical modification of the N- and/or C-termini (e.g., C-terminal amidation) may be used. The antiproliferative peptides of the invention possess detectable biological activity as inhibitors of proliferation of the predetermined cell population. It is apparent to those of skill in the art that variation of the precise amino acid sequence of antiproliferative peptides based on consensus anti-idiotype sequence motif(s) will effect the bioavailability, potency, efficacy, half-life, oxidation-resistance, solubility, and other physical, chemical, biological, and pharmacological properties that are relevant to formulating pharmaceutical compositions.

The amino acid sequences of anti-idiotypic peptides

identified by the methods of the invention will enable those of skill in the art to produce polypeptides corresponding to the anti-idiotype peptide sequences and sequence variants thereof. Such peptides may be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding an anti-idiotype peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides may be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and in vitro translation are well known in the art and are described further in Maniatis et al., Molecular Cloning: A Laboratory Manual (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA; Merrifield, J. (1969) J. Am. Chem. Soc. 91: 501; Chaiken I.M. (1981) CRC Crit. Rev. Biochem. 11: 255; Kaiser et al.(1989) Science 243: 187; Merrifield, B. (1986) Science 232: 342; Kent, S.B.H. (1988) Ann. Rev. Biochem. 57: 957; and Offord, R.E. (1980) Semisynthetic Proteins, Wiley Publishing, which are incorporated herein by reference).

For therapeutic or prophylactic uses, a sterile composition containing a pharmacologically effective dosage of one or more antiproliferative peptide is administered to a human patient or veterinary non-human patient for treatment of a lymphoproliferative condition. Generally, the composition will comprise a anti-idiotype peptide that is identical to or substantially similar to a peptide that binds specifically to the idiotype of a predetermined cell population that is the object of the therapy or prophylaxis. A pharmaceutically acceptable carrier or excipient is often employed in such sterile compositions. Routes of administration are typically intramuscular or intravenous injection or topical application, however some chemical forms of the invention may be effectively administered orally or by other routes. The compositions for parenteral administration will commonly comprise a solution of an antiproliferative peptide or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety

of aqueous carriers can be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of the antiproliferative peptide(s) in these formulations can vary widely, i.e., from less than about 0.01%, usually at least about 0.1% to as much as 5% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water, and about 10-1000 mg of antiproliferative peptide. A typical composition for intravenous infusion can be made up to contain 250 ml of sterile Ringer's solution, and about 100-1000 mg of antiproliferative peptide. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th Ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference. Excipients should be chemically compatible with the peptide(s) or peptidomimetic(s) that are the active ingredient(s) of the preparation, and generally should not increase decomposition, denaturation, or aggregation of active ingredient(s).

The antiproliferative peptides of this invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional peptides and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of biological activity

loss, and that use levels may have to be adjusted to compensate.

The compositions containing the present antiproliferative peptides or cocktails thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already affected by the particular lymphoproliferative disease, in an amount sufficient to cure or at least partially arrest the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose" or "effacacious dose." Amounts effective for this use will depend upon the severity of the condition, the general state of the patient, and the route of administration, but generally range from about 1 mg to about 2000 mg of antiproliferative peptide per dose, with dosages of from 10mg to 1000 mg per patient being more commonly used.

In prophylactic applications, compositions containing the antiproliferative peptides or cocktails thereof are administered to a patient not presently in a disease state to enhance the patient's resistance to recurrence or to prolong remission time. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 10 mg to 2000 mg per dose, especially 10 to 1000 mg per patient.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antiproliferative peptide of this invention sufficient to effectively treat the patient.

Individualized Antiproliferative Peptide Therapy

The methods of the invention are particularly suited to the rapid and inexpensive identification of antiproliferative peptides for treating individual human patients having lymphoma or lymphocytic leukemia. In general, a lymphocyte sample comprising neoplastic lymphocytic cells is obtained from a

patient, either as a blood or lymph sample or as a solid tumor biopsy, and used as a source of relevant immunoglobulin (or immunoglobulin superfamily molecule). The relevant immunoglobulin (or immunoglobulin superfamily molecule) is used to screen a peptide library and identify peptides which specifically bind; such peptides (or their substantially identical variants) are assayed for the ability to inhibit proliferation of the patient's neoplastic cells (obtained from a lymphocyte sample from the patient) in cell culture. Such peptides are identified as antiproliferative peptides and are administered to the patient in therapeutically effective dosage(s) for treating the lymphoma or lymphocytic leukemia.

Multiple anti-idiotype (antiproliferative) peptide sequences can be linked on a single peptide species for administration. For example and not limitation, ten antiproliferative peptide sequences may be identified for a particular lymphoma; polypeptides can be synthesized wherein each polypeptide comprises multiple copies of an antiproliferative peptide sequence and/or comprises more than one species of antiproliferative peptide sequence. For convenient reference, such peptides comprising multiple antiproliferative peptide sequences are termed "polyvalent peptides" or "repeat peptides". A polyvalent peptide which is comprised only of multiple copies of a single antiproliferative peptide sequence (plus any additional spacer or terminal amino acid sequences) is termed a homogeneous polyvalent peptide. A polyvalent peptide which is comprised of copies of more than one antiproliferative peptide sequence (plus any additional spacer or terminal amino acid sequences) is termed a heterogeneous polyvalent peptide. Polyvalent peptides typically comprise repeats of the antiproliferative peptide sequence(s).

Multimerization of Anti-Idiotype Peptides

Often, it is advantageous to form dimers or higher multimers of antiproliferative peptides. Frequently, antiproliferative peptides are dimerized (or form higher order multimers), either in peptide linkage or by other chemical

linkage (e.g., non-peptide covalent linkage, noncovalent linkage). Sometimes, a peptide containing two copies of an anti-idiotype peptide sequence are synthesized (with or without a spacer peptide sequence). In other embodiments, two anti-proliferative peptides are linked via a non-peptide linkage. Blondel and Bedoulle (1991) Protein Engineering 4: 457, report the design of a dimeric form of the maltose binding protein (MBP) that includes a 33-residue leucine zipper motif; other methods for dimerizing proteins also can be used to dimerize antiproliferative peptides, especially if the methods produce stable dimers. Ghadiri et al. (1992) J. Am. Chem. Soc. 114: 825, reports a method for assembling a small peptide into a multimeric structure by incorporation of a 2, 2'-bipyridine moiety in the peptide and the use of a metal ion to assist assembly.

The anti-proliferative peptides can comprise peptide motifs (association peptide sequences) that form tightly associated dimers and can be used to dimerize or otherwise aggregate the antiproliferative peptides. The association peptide sequences can dimerize when present in fusion proteins comprising the association peptide sequence and an antiproliferative peptide sequence added at the amino-terminus or the carboxy-terminus of the resultant fusion peptide. The dimerizable antiproliferative peptides of the invention are very stable, exhibit high binding affinities, and are useful in a wide variety of applications. In a preferred embodiment, the association peptide sequence is SKVILF and is fused to the amino-terminus of the anti-proliferative peptide sequence, optionally via a flexible linker or spacer such as GGPP, PPGG, or GGPPGG, or the like. In another preferred embodiment, the association peptide sequence is SKVILF, and is fused to the carboxyl terminus of the anti-proliferative peptide sequence by a linker or other attachment means so that a free carboxyl group is located in the resulting fusion peptide complex immediately following the F residue of the association peptide sequence. In an embodiment, a pair of association peptide sequences flank one or more copies of an anti-proliferative peptide sequence so that when the pair of association peptide sequences interact

intramolecularly, a loop is formed in the fusion peptide. In a similar fashion, one can bind two alpha-helical anti-proliferative peptides together side-by-side by placing association peptides at both ends of each alpha-helical polypeptide and forming intermolecular association peptide dimers of anti-proliferative peptides. In fact, anti-proliferative peptide comprising linked association peptide sequences can be polymerized to make linear polymers or polymeric gels.

Although the SKVILF peptide is a preferred association peptide sequence, other peptides, such as magainin peptide, metenkephalin, neurotensin, substance P, MHC peptide (see, Stagsted et al. (1990) Cell 62: 297), or the neuropeptide Y, which is a 36 amino acid peptide that can self-dimerize (see Cowley et al. (1992) Eur. J. Biochem. 205: 1099), can be used in the present methods. Heterologous association peptide sequences, i.e., two different peptides associate to form a heterodimer, such as "anti-sense" peptide sequences, can also be used to link anti-proliferative peptides (see Blalock and Smith (1984) Biochem. Biophys. Res. Comm. 121: 203; Shai et al. (1987) Biochem. 26: 669; Goldstein et al. (1989) Proc. Natl. Acad. Sci. USA 86: 42; Shai et al. (1989) Biochem. 28: 8804; and Lu et al. (1991) Proc. Natl. Acad. Sci. USA 88: 3642). In general, these association peptide sequences have one, two, or more copies of the motif defined by +XXX-, where "+" is a positively charged amino acid; "-" is a negatively charged amino acid; and each "X" can independently be any amino acid.

Thus, in one embodiment of the invention an antiproliferative peptide comprises an anti-proliferative peptide sequence in peptide linkage to an association peptide sequence; sometimes it is convenient to refer to these fusion peptides as associable anti-proliferation peptides. Typically, anti-proliferative peptides comprising association peptide sequences will form dimers (or higher multimers) linked by intermolecular bonds via interaction of the association peptide sequences.

For illustration and not limitation, an anti-proliferative peptide sequence may be identified as the sequence KPWYVTRV, and an associable anti-proliferative peptide comprising

this anti-proliferative peptide sequence could have the sequence KPWYVTRVSKVILF, SKVILFKPWYVTRV, KPWYVTRVGGPPSKVILF, SKVILFGGPPKPWYVTRV, KPWYVTRVPPGGSKVILF, SKVILFPPGGKPWYVTRV, KPWYVTRVGGPPGGSKVILF, SKVILFGGPPGGKPWYVTRV, or the like.

Alternative Embodiments

Optionally, a toxin may be conjugated, typically by covalent linkage, to an anti-idiotype peptide to deliver a toxic molecule (e.g., ricin, diphtheria toxin, phospholipase) to cells having surface immunoglobulin with substantially the same idiotype as the predetermined cell population.

For diagnostic embodiments, a detection agent such as a label (e.g., biotinyl moieties or FITC) can be linked to an anti-idiotype peptide or peptidomimetic. Similarly, metallothionein, a protein that binds heavy metal atoms, can be linked to, or expressed as a fusion protein with, an anti-idiotype peptide. The resulting product can be used to deliver radionuclides to cells having surface immunoglobulin with substantially the same idiotype as the predetermined cell population for imaging and therapy. Such diagnostic compositions may be used for histopathological diagnosis of neoplasms (e.g., for monitoring chemotherapy efficacy or recurrence of a lymphoma or lymphocytic leukemia) or other applications (e.g., localizing imaging or toxic agents to specific locations in the body for magnetic imaging or radioimaging in vivo or for cytotoxic effect). Such agents may include, for example, a linked component comprising: metals, chemotherapeutic drugs, radiosensitizing agents, cellular toxins, radionuclides, and others. Peptides can be labeled with ^{125}I or ^{131}I by the Bolton-Hunter method and by chloramine T.

The methods of the invention can be used to identify ligands of peptide-binding cell surface receptors other than members of the immunoglobulin gene superfamily. Such ligands may be antiproliferative peptides, pharmacological antagonists or agonists, or peptides which induce lymphocyte activation. For example, receptors which induce cell activation when antibodies bind to them are candidates for screening peptide libraries to

identify peptide ligands which inhibit cell proliferation, induce apoptosis, and/or modulate tyrosine phosphorylation (Ullrich and Schlesinger (1990) Cell 61: 203, incorporated herein by reference). The proto-oncogene products *her-2/neu* (Shepard et al. (1991) J. Clin. Immunol. 11: 117; Drebin et al. (1986) Proc. Natl. Acad. Sci. (U.S.A.) 83: 9129, incorporated herein by reference) and *fas* (Itoh et al. (1991) Cell 66: 233; Yonehara et al. (1989) J. Exp. Med. 169: 1747, incorporated herein by reference) are examples of such receptors. Such methods afford identification of antiproliferative peptides for inhibiting proliferation of lymphoid and non-lymphoid cells, such as neoplastic epithelial cells.

Kits can also be supplied for identifying anti-idiotype peptides which bind a predetermined cell population, such as a lymphoma biopsy sample, obtained from a patient. The kit components generally comprise a peptide library, typically in the form of a bacteriophage peptide display library or solid phase spatially defined peptide array (VLSIPS device), optionally including suitable host cells for propagating a bacteriophage display library, and a suitable aqueous binding buffer. Thus, the components for practicing the methods of the present invention may be provided, usually in a packaged form in a container, either alone or in conjunction with additional reagents. The bacteriophage particles or polynucleotide genomes are typically included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Typically, such kits will comprise an instruction manual for describing how to perform the screening method and an experimental protocol.

The following examples are offered by way of example and not by way of limitation.

EXPERIMENTAL EXAMPLES

Overview: The immunoglobulin superfamily molecules (e.g., IgM or IgD) of a lymphoma cell line or lymphocytic tumor specimen are produced by fusing the lymphoma cells to murine

myeloma cells and screening the hybridomas for the expression of the appropriate light and heavy chains. The immunoglobulins generally are purified from the hybridoma supernatants and used as the relevant immunoglobulin for screening a bacteriophage peptide display library by antibody panning. After four rounds of panning with relevant immunoglobulin, individual bacteriophage colonies were amplified and used in an ELISA with the relevant immunoglobulin to determine the relative or absolute binding affinity for individual selected bacteriophage clones to the relevant immunoglobulin. DNA from phage exhibiting high affinity binding to relevant immunoglobulin were sequenced to determine the encoded peptide sequence of the displayed peptide.

EXAMPLE 1: Antiproliferative Peptides to A Human Lymphoma

Purifying the relevant immunoglobulin: A cell suspension obtained from tumor biopsy specimens or from lymphoma cell lines were isolated as sources of predetermined cell populations. The cell suspensions were mixed with HAT-sensitive heterohybridoma B5 cells. B5 is a heterohybridoma that was generated by the fusion of the NS-1 murine fusion partner (American Type Culture Collection, Rockville, MD) with a human lymphoma cell. The B5 clone has lost the ability to secrete Ig spontaneously. A subclone which retains the ability to secrete Ig when fused to with human B cells. This subclone was made HAT-sensitive by growth in medium containing 8-azaguanine. The mixture of lymphoma cells and B5 cells is exposed to 40% polyethylene glycol and washed with PBS. The cells were plated at a density of 2×10^5 cells/well in HAT medium into 96-well microtitre plates. The cells were screened by ELISA for secretion of immunoglobulins of the same light chain class as the lymphoma. The highest producing clones are expanded to larger culture volumes. The supernatants of the hybridomas are pooled and run through protein A columns (for IgG-secreting hybridomas) or a Sepharose column coated with a monoclonal anti-IgM antibody (for IgM-secreting hybridomas). The Ig is eluted from the column with glycine-HCl and dialyzed overnight in PBS. The resultant dialyzed Ig constitutes relevant immunoglobulin with respect to

the predetermined cell population used to generate it. A relevant immunoglobulin from a B cell lymphoma of a patient was so prepared and used to screen three bacteriophage peptide display libraries. The cell line was identified and designated Sup B8 which expresses a monoclonal immunoglobulin designated as the Tab idiotype; the Tab monoclonal antibody was used to screen peptide libraries to identify anti-idiotype peptides.

Filamentous phage display libraries: The vector pAFF1 and the libraries used are available from Affymax, Palo Alto, California. A description of the construction of one of the libraries follows: The vector has two BstXI sites at the 5' region of gene III. The vector is cut with BstXI and cut vector is purified. A collection of oligonucleotides encoding all possible octapeptides with the sequence 5'C TCT CAC TCC $(NNK)^8$ GGC CTG GTT CCG CGT GGA TCC GGC ACT GTT GAA AGT TGT 3' in which N stands for equimolar A, C, G, and T, and K stands for equimolar ratios of G or T, is annealed to two "half-site" oligonucleotides with the sequences 5'-GGA GTG AGA GTA GA-3' and 5'-CTT TCA ACA GT-3' and ligated into the vector by the addition of ligase and ATP. The single stranded gaps are filled in with Klenow DNA polymerase. The DNA is electroporated into E. coli MC1061 in multiple batches. The electrotransformations are pooled and grown in selective media through 10 doublings. The phage are isolated by clearing the supernatant from the bacteria by centrifugation, followed by precipitation of the phage by polyethylene glycol. The phage were plated out on LB-tetracycline plates to determine the titer of infectious particles.

Identifying ligands with high affinity with the immunoglobulin receptor

The purified Ig's were immobilized on plastic plates or sepharose columns. Approximately 10^{11} to 10^{12} infectious phage are incubated with the Ig. The plates or the column is washed with a wash buffer. Adherent phage are eluded with glycine-HCl. Eluted phage are amplified by infecting logarithmic E. coli K91 and growing them in selective media overnight. The

phage were isolated from the plates, separated from the bacteria, purified by polyethylene glycol precipitation, resuspended, and amplified. The affinity purification step was repeated four times. After the final round, E. coli is infected with the phage and plated at low density. Individual colonies were picked and amplified in E. coli overnight. Phage were purified from bacterial supernatant. A phage ELISA was performed on the amplified individual phage to determine the binding to the given immunoglobulin immobilized on the ELISA plate. The genomes of those phage which preferentially bound to the relevant immunoglobulin were sequenced. Single stranded DNA was prepared from the phage and sequenced with a primer complementary to the sequence of gene III, 15 nucleotides to the 3' side of the BstXI site using the chain-termination DNA sequencing method. A peptide with the amino acid sequence corresponding to a common nucleotide sequence of phage obtained from affinity purification was made synthetically and purified by HPLC. A variant peptide elongated at the carboxy-terminus by the amino acid sequence G-G-L-R-R-A-S-L-G, the substrate for cAMP-dependent protein kinase, was also synthesized. The affinity constant of the peptide ligand is determined with soluble radioimmunoassay using the ^{32}P labeled variant peptide and the purified Ig. Fig. 1 shows inhibition of anti-idiotype antibody binding to the relevant immunoglobulin with anti-idiotype peptide 3T802 with an IC_{50} of between 1 and 10 μM. Immunoglobulin of the Tab idiotype was plated on 96-well microtitre plates and detected with murine monoclonal anti-idiotype antibody in an ELISA format and the ability of the peptide to inhibit binding of the anti-idiotype monoclonal to Tab was measured. Binding was measured with peptide added prior to the addition of the anti-idiotype antibody.

Proliferation assays of lymphoma cells with and without ligand were performed in 96 well tissue culture plates. 5,000 to 10,000 tumor cells per well are plated in 100 μl complete medium. Peptides, biotinylated peptides, biotinylated peptides with streptavidin were reconstituted in 100 μl of medium and added to the wells. The cells were incubated for 3 days at 37

degrees Celsius. XTT assays (Jost et al. (1992) J. Immunol. Meth. 147: 153, incorporated herein by reference) were performed to quantitate the number of cells proliferating at that time. Fig. 2 shows the antiproliferative effect of biotinylated peptide 3T802 conjugated to streptavidin. In a three day proliferation assay, approximately 5,000 Tab lymphoma cells were plated on complete medium mixed with peptides in 96-well culture plates. After three days, an XTT assay was performed measuring the number of metabolically active cells. Data is presented as averages from five samples in each group.

An alternate method for quantitation of proliferation is ^{3}H-thymidine incorporation assays. ^{3}H-thymidine is added 24 hours before cell harvest, followed by cell harvest onto glass fiber filters and determination of incorporated radioactivity with a liquid scintillation counter.

Immunoblots were used to evaluate the stimulation of protein tyrosine phosphorylation by ligand binding. Cells growing at mid-log phase are washed in fresh medium. About 2 million cells are reconstituted in 1 ml medium. Anti-IgM antibodies or peptides are added for various time periods. Phosphorylation is then inhibited with a buffer containing 1 mM sodium orthovanadate in PBS. The cells are lysed. The lysates are run on an SDS-polyacrylamide gel and subsequently transferred to nitrocellulose. Phosphorylated tyrosine are detected with a monoclonal anti-phosphotyrosine antibody.

Antiproliferative peptides: The relevant immunoglobulin from a B cell lymphoma of a human patient was used to screen three bacteriophage display libraries. The amino acid sequences of about at least 60 positive phage which bound to the relevant immunoglobulin were identified by sequencing the peptide display portion of the pIII gene of the selected phage. Several of the peptides were produced by chemical synthesis. The peptides were determined to bind to immunoglobulin of the predetermined cell population (B cell lymphoma) obtained from the patient but not to those of other B lymphocytic cell types tested. The anti-idiotype peptides were biotinylated and linked with streptavidin

by allowing 50 μM streptavidin reconstituted in RPMI to react with 200 μM biotinylated anti-idiotype peptide in a 1:1 mixture over 30 minutes at room temperature to produce a tetramer of four peptides conjugated to a streptavidin molecule and was shown to inhibit the growth of the predetermined cell population (i.e., a cell line established from the patient's B cell lymphoma cells) but not other B lymphocytic cell lines with different idiotypes. The anti-idiotype peptide streptavidin complex induced tyrosine kinase activity in the cell line established from the patient's B cell lymphoma cells but not in other B lymphocytic cell lines with different idiotypes.

Table I shows amino acid sequences of some of the peptides identified and shown to bind the idiotype of the B cell lymphoma SUP-B8.

Table I

Sample-ID	Sequence
MR401	K-P-W-Y-V-S-R-V
4R02	K-P-W-Y-V-G-R-P
4R09	K-P-W-Y-V-T-R-V
3T813	K-P-W-W-V-T-R-V
3T802	K-P-W-W-V-S-R-V
4R07	K-P-W-W-V-V-R-L
MR311	G-K-P-W-W-A-S-R
4R14	E-K-P-W-W-A-V-R
MR304	G-K-P-I-W-A-G-R
MR421	K-P-S-N-V-S-R-V
MR426	V-P-W-Y-K-Q-S-T
MR430	A-P-W-Y-R-V-S-P
MR440	L-P-W-Y-L-Y-P-S
MR442,446	G-K-P-W-Y-A-G-R
3T821	Q-K-P-I-W-V-T-R
MR416	S-P-W-Y-R-W-H-N
3T1204	K-N-G-P-W-Y-A-Y-T-G-R-D
MR432,35	K-W-Y-K-E-R-W-N
MR402	S-W-Y-D-R-V-W-D
MR411	L-W-Y-D-D-P-W-P
MR408	W-W-Y-D-E-V-W-G
MR412	A-W-P-N-E-M-Y-V
MR431	H-W-Y-N-E-Y-W-D
MR418	G-W-Y-N-E-T-W-H
MR422,23,28 3	Y-Y-C-S-P-W-C-D
MR307	S-W-Y-N-D-W-F-P
3T1206	S-V-P-P-A-W-Q-S-R-V-W-N
3T1220	S-W-Y-D-Q-V-W-W-D-S
3T1217	R-S-P-S-H-W-Y-K-E-M-W-D
MR427	Q-V-W-Y-K-W-P-N
MR416	S-P-W-Y-R-W-H-N
MR444	I-V-P-W-Y-R-W-T
MR430	A-P-W-Y-R-V-S-P
MR413	V-S-I-E-W-Y-R-F
T812	D-W-A-V-W-N-R-R
4R01	N-W-A-V-W-T-K-R
T817	N-W-G-M-W-S-K-R
T807	S-E-P-V-D-H-G-L
T811	V-D-P-V-D-H-G-L
T819	V-P-I-D-H-G-T
4R16	K-P-A-W-V-T-R-Q
1206	S-V-P-P-A-W-Q-S-R-V-W-N
T807	G-W-S-P-F-I-A-M
4811	G-W-V-P-F-I-S-L
MR436	Y-F-H-S-M-H-V-R
MR305	F-H-N-A-S-G-S-G
T801	Y-S-F-W-D-L-V-K
T804	M-P-E-D-F-Y-R-R
T805	C-W-T-A-D-C-K-V
T808	G-L-M-E-M-V-R-R
T809	Y-E-M-P-E-Y-K-R
T810	K-Y-R-L-C-Q-V-C
T816	Y-V-F-E-D-L-F-R
T822	H-W-A-A-L-M-K-R
4R03	M-P-E-D-F-Y-R-R
4R06	R-F-E-S-M-F-K-R
4R10	Y-W-E-A-H-V-R-R
4L09	V-F-W-Q-M-I-R-R
4L19	M-P-W-A-M-F-R-R
4L29	S-F-M-D-M-F-K-R

EXAMPLE 2: Antiproliferative Peptides to the Transplantable Human B-Cell Lymphoma Line SUP-B8

This example further describes peptide ligands found by screening phage display libraries for binding to the purified IgM,λ receptor of the human Burkitt's lymphoma cell line SUP-B8 (Wright et al. (1989) J Exp Med 169:1557; Carroll et al. (1988) Blood 71:1068). The antiproliferative peptides' cytotoxic activity(ies) and effect(s) on signal transduction and cell death in vitro and in vivo is demonstrated.

Overview

Peptide ligands for the surface immunoglobulin receptor (Ig) of a human B-cell lymphoma cell line were identified with the use of filamentous phage libraries displaying random 8 and 12 amino acid peptides. Corresponding synthetic peptides bound specifically to this Ig receptor, and blocked the binding of an anti-idiotype antibody. The ligands, when conjugated to form dimers or tetramers, induced cell death by apoptosis in vitro at nanomolar concentrations. This effect was associated with the specific stimulation of intracellular protein tyrosine phosphorylation.

The experiments described in this example are an extension of Example I (*supra*).

Material and Methods

Cell lines

The B-cell lymphoma cell lines SUP-B8 (Carroll et al. (1988), op.cit) SU-DHL4 (Kaiser-McGaw et al. (1985) Cancer Genetics and Cytogenetics 14:205) and OCI-Ly8 (Tweeddale et al. (1987) Blood 69:1307) were cultured as published. A mouse monoclonal anti-idiotype (anti-Id) antibody to the SUP-B8 Ig was produced as published (Carroll et al. (1988), op.cit). The following E. coli bacterial strains were used: K91, MC 1061 F, DH5αF'.

Immunoglobulin receptor purification

SUP-B8 cells, SU-DHL4 cells, or tumor cells from patients were fused with the heterohybridoma K6H6/B5 and screened

for the production of IgM/λ. The Ig was affinity purified as published (Carroll et al. (1988) op.cit; Kwak et al. (1992) N Engl J Med 327: 1209).

Random peptide phage libraries

Three phage libraries were constructed as published (Cwirla et al. (1990) op.cit) displaying NH_2-terminal random octapeptides linked to pIII with the spacers ASASA (single letter amino acid code) (Library 1), or polyproline (mixture of P_4 and P_6) (Library 2), or GG in the 12-mer library (Library 3).

Affinity Selection using phage libraries (panning)

SUP-B8 Ig was immobilized on 96-well microtiter plates by adding 0.5 μg Ig to each well. Plates were washed and blocked essentially as described (Scott JK and Smith GP (1990) op.cit; Wright et al. (1989) op.cit). Transducing units (TU) of bacteriophage in 600 μl Tris-buffered saline (50mM Tris-HCl, pH 7.5, 150 mM NaCl) were distributed equally among 6 wells and incubated for 2 hours at 4°C. The plates were washed with PBS. Bound phage were eluted with 0.1M glycine HCl, pH 2.2, neutralized with 2M Tris base, amplified in K91 cells, and precipitated for further rounds of panning (Cwirla et al. (1990) op.cit). In the third and fourth round of panning, 1/10 and 1/100 respectively, of the number of phage used in round 1 were applied to the Ig. Individual phage clones were grown, purified, characterized by phage ELISA (see *infra*) and subjected to DNA sequencing.

Mutagenesis phagemid libraries

Three independent mutagenesis libraries were constructed in a manner similar to the random libraries (Cwirla et al. (1990) op.cit) except that they were cloned into a pIII phagemid vector (pAFF2MBP) (Cull et al. (1992) Proc Natl Acad Sci USA 89:1865; Lee, N. (1980) The Operon pp. 389-409). In this phagemid system, recombinant pIII expression was regulated by the inducible ara-B promoter. Recombinant pIII was incorporated into phage particles along with wild type pIII provided by helper

phage (Lowman et al. (1991) Biochemistry 30: 10832). The oligonucleotides encoding the pIII inserts for the libraries were synthesized as follows: For each base addition, denoted in lower case letters below, 70% was the "correct" base, encoding the amino acid of the input sequence, and the remaining 30% were split equally among the three remaining bases. If the consensus sequence had two amino acids at a position, the base composition was 40% each of the bases encoding the consensus (denoted by r=AC, s=GC, w=AT, y=TC), and 10% each of the two remaining bases. Positions with no preferred residue were left random with the sequence NNK, where N=A,C,G,T and K=G,T. The mutagenesis library oligonucleotides were synthesized (A library: 50% C TCT CAC TCC aag ccN tgg gyN NNK agg gtN GGC ACT GTT GAA AGT TGT; 50% C TCT CAC TCC aag ccN tgg tat gyN NNK agg gtN GGC ACT GTT GAA AGT TGT. B library: C TCT CAC TCC ray tgg gsN rtg tgg NNK agg GGC ACT GTT GAA AGT TGT. C library: C TCT CAC TCC tat NNK gag gaN wtg tat agg GGC ACT GTT GAA AGT TGT), gel purified and enzymatically phosphorylated.

Each library was constructed by annealing 3 oligonucleotides with BstX I-digested and purified vector as in the random libraries in the following ratios: 1.6 pmoles of pAFF2MBP/4.1 pmoles of library oligo/4.1 pmoles of GGAGTGAGAGTAGA/82 pmoles of CTTTCAACAGT. The gapped plasmid was filled in by the addition of dNTP and T7 DNA polymerase and incubation for 90 minutes at 37°C. The ligated filled plasmid was precipitated and resuspended. The DNA was electroporated into MC1061F cells and grown in LB/Ampicillin (100 μg/ml)/0.1% $MgSO_4$/0.25% KH_2PO_4 buffered medium containing 0.1% glucose for pIII repression. At early logarithmic phase (A_{600} of 0.3), the cultures were infected with VCSm13 helper phage. Phagemid producing cells were then induced and selected, respectively, by the addition of 0.02% arabinose and kanamycin, (20 μg/ml). Phagemid were precipitated with polyethylene glycol/NaCl (Cwirla et al. (1990) op.cit), resuspended in PBS/0.1% BSA, and stored at -20°C. Each mutagenesis library was panned independently as previously described for the random libraries. Eluted phage were amplified by infection of DH5αF' cells.

Phage ELISA

Binding of phage to immobilized Ig's was measured in an ELISA format essentially as published (Barrett et al. (1992) Anal Biochem 204:357). Competition ELISA was performed by adding monoclonal anti-Id antibody in at varying concentrations to the wells to compete with the binding of phage to SUP-B8 Ig.

Peptide Synthesis, Purification and Polymerization

A peptide derived from one consensus sequence containing clone from each mutagenesis experiment was synthesized and HPLC purified to 95% purity by the PAN Facility at Stanford University or by Neuros, San Jose, CA. The peptide content was confirmed by amino acid analysis, and the composition was verified by mass spectroscopy. All peptide monomers were synthesized with the C-terminal extension GGK-Biotin, with the ϵ-amino group of lysine biotinylated. The amino-acid sequences of the peptides synthesized were: Peptide A: (KPWWVSRVGGK-Biotin), Peptide B (DWAIWSKRGGK-Biotin), and Peptide C (YSFEDLYRRGGK-Biotin). The following three control peptides were synthesized: a peptide with the reverse amino acid sequence of one of the nonspecifically binding phage: Control Peptide D (Biotin-KSADWVRDYWS), a peptide with a randomly scrambled amino acid sequence of Peptide C: Scrambled Peptide C (RDYSYERLFGGK-Biotin) and a peptide with the reverse sequence of Peptide C: Reverse Peptide C (Biotin-KGGRRYLDEFSY). Peptide A was also synthesized with the C-terminal extension SSC (KPWWVSRVSSC) for dimerization. A random repeat peptide of Peptide C was synthesized with the amino-acid sequence of Peptide C incorporated twice, separated by 6 glycine residues: Random Repeat Peptide C (YSFEDLYRRGGGGGGYSFEDLYRR). A control tandem repeat peptide was synthesized incorporating the scrambled sequence of Peptide C: Tandem Repeat Scrambled Peptide C (RDYSYERLFGGGGGGRDYSYERLF).

The variant of peptide A with a C-terminal cysteine (KPWWVSRVSSC) was incubated at room temperature for 24 hours in 2M Tris, pH 8.5 to allow the oxidation of the sulfhydryl groups to form dipeptides. To form tetramers, equal volumes of 50 μM

streptavidin and 200 μM biotinylated peptide monomer were mixed and rotated for 30 minutes at room temperature.

Peptide ELISA

Biotinylated monomer peptides were immobilized on microtiter plates coated with streptavidin: 1 μg Streptavidin was added to each well of a microtiter plate and incubated overnight at 4°C. After washing and blocking with PBS/5% nonfat dry milk, SUP-B8 Ig or control Ig was added at 5 μg/ml. Binding of Ig was detected with goat anti-human lambda/horseradish peroxidase (HRP) conjugate or goat anti-human kappa/HRP conjugate (Tago, Burlingame, CA). In the anti-Id peptide competition assay, 0.5μg SUP-B8 Ig was immobilized on a microtiter plate. Peptides were added at the shown concentrations. After 30 minutes, anti-Id antibody was added to each well, and detected with goat anti-mouse IgG/HRP conjugate.

Proliferation Assays

$5x10^3$ cell per well were plated in 96-well tissue culture plates and incubated with peptide tetramers, dimers or tandem repeat peptides at the shown final concentrations for 72 hours. Cell proliferation was measured by adding 1μCi ^{3}H-thymidine (Amersham) per well 48 hours into the experiment. 24 hours later, cells were lysed with water and harvested onto glass filters. ^{3}H-thymidine incorporation was measured by liquid scintillation counting. All data shown are means of raw cpm.

Phosphotyrosine Assay

Cell lysates were electrophoresed under reducing conditions on an 8% SDS-PAGE gel, electrotransferred to nitrocellulose, and probed with goat anti-mouse IgG-Biotin antibody alone to show equal loading of cell lysates into all wells. After washing, the blot was probed with the monoclonal anti-phosphotyrosine antibody 4G10, detected with goat anti-mouse IgG-Biotin and visualized with streptavidin/HRP as published (Schick et al. (1993) J Immunol 151:1918).

Fig. 3 shows a Western blot showing tyrosine

phosphorylation in SupB8 (Tab) cells. Approximately 2 x 10^6 SupB8 (Tab) cells were stimulated with peptides (10 μM) or antibodies. After 2 minutes or the times shown in Fig. 3, cells were washed in buffer containing orthovanadate to inhibit phosphorylation. Cell lysates were electrophoresed using an 8 % SDS-PAGE and transferred to nitrocellulose. In panel (A), phosphorylated tyrosines were detected with a murine anti-phosphotyrosine antibody and visualized with a goat anti-mouse IgG antibody conjugated to horseradish peroxidase. In panel (B), detection with the goat anti-mouse IgG antibody coupled to horseradish peroxidase alone, showing equal loading in all lanes. The blot shows active phosphorylation with the anti-idiotype peptide (lanes 6-10) as early as 1 minute after stimulation, but not with a control peptide-streptavidin complex (lane 5).

Apoptosis Assay

2.5x10^6 SUP-B8 cells were cultured with 2.5 μM peptide tetramers or tandem repeat peptides for 24 hours. Cells were washed in PBS once, resuspended in 50 μl PBS, and entrapped into agarose by mixing with 100 μl melted 2% InCert agarose (FMC Bioproducts, Rockland, ME). The mixture was poured into a plug-forming mold and cooled at 4°C for 30 minutes. The gel plugs were removed from the mold and treated overnight at 50°C with 100mM EDTA pH 8.0, 1% Sarkosyl, 1 mg/ml proteinase K. The following day the plugs were treated for 2 hours at 50°C with RNAse A, 0.1 mg/ml in 10 mM Tris, 0.1 mM EDTA, pH 8.0. The gel plugs were inserted into a 1.5% agarose/TBE gel, electrophoresed and stained with ethidium bromide.

RESULTS

Three different phage libraries, each containing approximately 5x10^8 random 8-mer or 12-mer peptides, were subjected to four rounds of panning on the Ig of SUP-B8. Phage from individual clones were purified. Their binding to the SUP-B8 Ig and to control Igs was tested in an ELISA format. Specificity was further tested by inhibiting the binding of the phage with a mouse monoclonal anti-Id antibody. After four

rounds of panning, nearly all phage clones bound specifically. Their binding to the SUP-B8 Ig could in most cases be inhibited with anti-Id antibodies, but not with anti-IgM or anti-λ light chain antisera. Those phage clones that bound specifically to SUP-B8 were subjected to DNA sequencing. Amino acid sequences of peptides from these phage are shown in Table I (*supra*) and in Table II, which groups the amino acid sequences on the basis of sequence identity. The peptide sequences fell into four groups of homology (groups A-D). Within group A and group C, phage were isolated from two libraries, suggesting that the sequences retrieved were independent of the different linkers used to link the random peptides to pIII in the phage constructs. Even though a consensus sequence could be determined for each of the four homology groups, some positions were poorly conserved.

Table II

Group	Library	Peptide Sequences
A	1	K P W Y V S R V
	2	K P W Y V G R P
	2	K P WW V T R V
	2	K P WW V S R V
	2	K P WW V V R L
	1	G K P WW A S R
	1	E K P WW A V R
	1	G K P W Y A G R
	2	G K P I W A G R
	1	K P S N V S R V
	1	Y Y C S P W C D
	1	V P W Y K Q S T
	1	A P W Y R V S P
	1	L P W Y L Y P S
	2	Q K P I W V T R
	3	K N G P W Y A Y T G R D
	Consensus:	K P W W/Y V/A x R V
B	2	D W A V W N R R
	2	N W A V W T K R
	2	N W G M W S K R
	Consensus:	N/D W A/G V/M W x R/K R
C	2	Y V F E D L F R
	2	M P E D F Y R R
	3	F G I L T E E M Y R R W
	3	L R Y T Q E E M Y R R W
	3	H Y V H E D L Y R R V K
	3	V T G Y T M D V L Y R R
	Consensus:	Y x x E D L Y R R
D	2	S E P V D H G L
	2	V D P V D H G L
	2	V P I D H G T
	Consensus:	x x P V D H G L

In order to define the critical residues in each peptide family, three new phagemid mutagenesis libraries were generated based upon groups A-C of Table II. These libraries were constructed such that the oligonucleotides encoding the pIII insert were synthesized as a family of oligonucleotides based upon the DNA sequence of the consensus peptides, varied to a defined degree. The amino acid composition of the resultant libraries differed by approximately 50% from the consensus sequence. The library size for all three libraries was approximately 10^8 TU. After three rounds of affinity selection, phage clones binding to SUP-B8 Ig were identified and subjected to DNA sequencing. Table III summarizes the results, showing the sequences towards which the three libraries were biased (input) and the frequency of amino acids obtained after selection (output). The consensus sequences determined from screening random libraries were mostly confirmed by screening the mutagenesis libraries. Some exceptions were the emergence of I for V/M in the fourth position of mutagenesis library B, or K for R in the seventh position at the C-terminus of the same group. Other poorly conserved positions from the random library screen were defined better, such as the 6th position of library A. Based on these results, a peptide from each mutagenesis consensus sequence was synthesized.

Table III

Mutagenesis Library A:

Input:	K	P	W	W/Y	V/A	X	R	V
Output: N=20	K=20	P=19 S=1	W=18 G=1 Q=1	Y=10 W=8 F=1 S=1	V=18 A=2	S=14 T=3 L=1 A=1	R=18 G=2	V=11 L=4 I=3 A=1 G=1
Consensus:	K	P	W	W/Y	V	S	R	V
Peptide A:	K	P	W	W	V	S	R	VGGK(Biotin)

Mutagenesis Library B:

Input:	N/D	W	A/G	V/M	W	X	R	R
Output: N=27	D=23 N=3 K=1	W=27	A=16 G=10 P=1	I=12 V=8 L=7	W=26 G=1	S=13 N=7 M=6 R=1	K=27	R=27
Consensus	D	W	A	I	W	S	K	R
Peptide B:	D	W	A	I	W	S	K	RGGK(Biotin)

Mutagenesis Library C:

Input:	Y	X	X	E	D/E	L/M	Y	R	R
Output: N=24	Y=24	S=8 T=4 V=4 F=2 A=2 Q,Y=1 E,G=1	F=14 I=4 L=4 M=1 Y=1	E=20 Q=3 G=1	D=20 E=2 S=1 A=1	L=22 M=2	Y=23 F=1	R=19 K=5	R=24
Consensus:	Y	S	F	E	D	L	Y	R	R
Peptide C:	Y	S	F	E	D	L	Y	R	RGGK(Biotin)

The synthesized peptides A-C bound specifically to the SUP-B8 Ig and not to control Ig receptors from other B-cell lymphomas or to normal polyclonal IgM (Fig. 4). The control peptides with the scrambled or reverse amino acid sequence of peptide C did not bind to SUP-B8 Ig. Additionally, Peptides A-C inhibited the binding of the bivalent anti-Id to SUP-B8 Ig (Fig. 5), while the Control Peptide D did not. None of the peptides inhibited the binding of anti-IgM or anti-λ antisera to SUP-B8 Ig.

The peptide monomers themselves had little or no effect on the proliferation of SUP-B8 cells in vitro (Fig. 6, panel A). Thus, crosslinking of the surface Ig receptors is required to induce a substantial anti-proliferative effect in B-cell lymphoma cell lines. Monomeric F(ab) fragments of anti-Ig antibodies fail to inhibit cell proliferation (Udhayakumar et al. (1991) J Immunol 146: 4120). To crosslink surface Ig receptors, peptide polymers were made by reacting biotinylated peptides with tetravalent streptavidin. The resulting putative peptide ligand tetramers were reproducibly cytotoxic, with an IC_{50} of 60 to 200 nM (Fig. 6, panel B). After three days, only cell fragments were seen in the treated wells. The Control Peptide D tetramer, Scrambled Peptide C tetramer and Reverse Peptide C tetramer had no apparent effect on the cells. Streptavidin alone did not inhibit proliferation (Fig. 6, panel A). The peptide ligand tetramers had no influence upon the proliferation of the control B-cell lymphoma cell lines OCI-Ly8 (Fig. 6, panel C) and SU-DHL4, indicating that the cytotoxic effect of the peptides was tumor specific.

Peptides containing C-terminal cysteines were oxidized to form dimers through disulfide bridges. The dimer could be reduced with 2-mercaptoethanol to monomers, as confirmed by HPLC analysis. A cytotoxic effect similar to the effect of the tetramers was seen with the peptide ligand dimer (Fig. 7, panel A). A control peptide subjected to the same dimerization procedure had no effect. A tandem repeat form of Peptide C was synthesized as a 24-mer peptide incorporating the sequence of Peptide C twice, separated by six glycine residues. A control

tandem repeat dimer with a randomly scrambled sequence of Peptide C was synthesized as well. The Random Repeat Peptide C was as cytotoxic to SUP-B8 cells as the tetramers (Fig. 7, panel B), with an IC_{50} of about 40 nM. The Tandem Repeat Scrambled Peptide C did not inhibit proliferation of SUP-B8 cells. The Tandem Repeat Peptide C had no effect on the proliferation of the control B-cell lymphoma cell lines OCI-Ly8 (Fig. 7, panel C) and SU-DHL4.

The cytotoxic effect of the peptide ligand tetramers was reproducibly associated with specific phosphorylation of intracellular protein tyrosines. A pattern of tyrosine phosphorylation comparative to that seen with anti-IgM antiserum (Fig. 3, lane 2) occurred within 1 minute of exposure of SUP-B8 cells to Peptide A (lanes 4-8). No increase in protein tyrosine phosphorylation was seen with the Control Peptide D tetramer (lane 3) or with streptavidin alone. There was no effect on protein tyrosine phosphorylation of the irrelevant OCI-Ly8 cells with Peptide A tetramers. Fig. 3, panel B shows a non-specific protein with a 76 kDa apparent molecular weight as well as the Ig heavy chain of SUP-B8 cells, detected in the same blot as Fig. 3, panel A with a goat anti-mouse IgG antibody, demonstrating equal loading of all lanes.

The SUP-B8 cells underwent programmed cell death when stimulated with the peptide tetramers. DNA fragmentation, a characteristic feature of cells undergoing apoptosis, was seen after 24 hours of incubation with Peptide A-C tetramers, but not with the Scrambled Peptide C tetramer or Reverse Peptide C tetramer (Fig. 8). DNA fragmentation was not seen in the unrelated OCI-Ly8 cells incubated with Peptides A-C tetramers or the control peptide. Similarly, the Tandem Repeat Peptide C and not the Tandem Repeat Scrambled Peptide C caused DNA fragmentation.

These experiments demonstrate the application of phage display libraries in the discovery of surrogate ligands to a cell surface receptor. Several groups of surrogate ligands were found. Within each group, some amino acid residues were conserved, while others were not. Screening mutagenesis

libraries helped to further define the critical residues. However, the consensus sequences derived from screening mutagenesis libraries were not greatly different from the consensus sequences obtained from the random library screen. Thus, it may not be necessary to screen mutagenesis libraries in all cases.

This previously undescribed specific cytotoxic activity of peptides, seen in the absence of effector cells or complement, provides the basis for a new form of therapy for lymphoma and other lymphoproliferative disorders characterized by an expansion of a clonal population of lymphocytic cells wherein each member of the clonal population bears a surface immunoglobulin superfamily molecule of the same idiotype. The cytotoxic activity of the antiproliferative peptides is mediated through the transmembrane Ig receptor as demonstrated by the rapid and specific increase in tyrosine phosphorylation after exposure to the peptide ligand tetramers, followed by apoptosis. Evidence of signal transduction induced by antibodies against the Ig receptor on human lymphomas, evaluated by the degree of tyrosine phosphorylation in vitro as described *supra*, predicts which patients will respond clinically to anti-idiotypic therapy. This provides a tool to evaluate the potential therapeutic benefit of surrogate peptide ligands in vitro. In some embodiments, the incorporation of D-amino acids or other structural modifications of the peptide can be employed to enhance pharmacokinetic and/or pharmacological properties (e.g., stability).

In Vivo Testing of Antiproliferative Peptide Therapy

Human lymphomas established in SCID mice (Schmidt-Wolf et al. (1991) J Exp Med 174:139) are known in the art as highly predictive models for studying human lymphoma biology and evaluating therapeutic agents and treatment modalities. As such, human lymphoma lines established in SCID mice provide a suitable in vivo model to test the therapeutic efficacy of synthetic peptide ligands. Indeed, the SUP-B8 tumor can be propagated in SCID mice and afford the in vivo demonstration that Peptides A-C or their derivatives can act as anti-lymphoma drugs.

SCID mice were irradiated with 200 cGy whole-body irradiation. One day later, the mice were injected with 1×10^7 SUP-B8 human tumor cells intravenously into the tail veins. Animals were kept under sterile conditions and water was supplemented with Septra for four days of the week. All mice developed detectable neoplastic disease and SUP-B8 immunoglobulin receptor was detected in all mice by Day 37. The mice were split into three groups. One group was not treated with any agent (Control group, 10 mice). A second group (7 mice) was injected intravenously into tail veins with Tandem Repeat Peptide C (*supra*) with 200 μl of a 200 μM solution in sterile saline on the following day (Day 38), as well as on Days 42, 45, 48, and 52. A third group of mice was injected with a single intravenous injection of 200 μl of 100 μM Peptide C Tetramer in sterile saline on Day 38. The mice were observed for survival and survival curves were measured (Fig. 9). As is shown in Fig. 9, mice injected with antiproliferative peptide have detectably prolonged survival times and have a significantly reduced susceptibility to death from the transplanted human lymphoma.

Although the present invention has been described in some detail by way of illustration for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.

CLAIMS

1. A method for identifying antiproliferative peptides, comprising the steps of:

obtaining a predetermined cell population from a patient, wherein said predetermined cell population comprises cells expressing on their extracellular surface an immunoglobulin superfamily species having a single idiotype characteristic to the predetermined cell population;

contacting under aqueous binding conditions said immunoglobulin superfamily species to a peptide library comprising a multiplicity of peptide library members having distinct peptide sequences;

identifying a peptide library member that binds specifically to said immunoglobulin superfamily species idiotype as an anti-idiotype peptide;

contacting under growth conditions said anti-idiotype peptide to said predetermined cell population or their clonal progeny and measuring an indicator of cell proliferation or activation in the predetermined cell population; and

identifying an anti-idiotype peptide which inhibits cell proliferation of the predetermined cell population as an antiproliferative peptide.

2. A method according to claim 1, comprising the further step of determining the amino acid sequence of said anti-idiotype peptide.

3. A method according to claim 1, wherein the peptide library is a bacteriophage display library comprising a multiplicity of bacteriophage species having sequences of said peptide library members displayed as a fusion proteins with a coat protein of the bacteriophage.

4. A method according to claim 1, wherein the immunoglobulin superfamily species is an immunoglobulin expressed by a B cell lymphocytic cell population.

5. A method according to claim 1, wherein said B cell lymphocytic cell population is a human B cell lymphoma.

6. A method according to claim 1, wherein the indicator of cell proliferation is tyrosine phosphorylation of a polypeptide comprising tyrosine.

7. A method of claim 1, wherein the anti-idiotype peptide is a peptide sequence shown in Table I or Table II.

8. A method of claim 7, wherein the antiproliferative peptide is peptide 3T802 and the predetermined cell population is Sup B8.

9. A method of claim 1, wherein the anti-idiotype peptide sequence is not less than 8 and not more than 12 amino acids in length.

10. An anti-idiotype peptide identified by the method of claim 1.

11. An anti-idiotype peptide of claim 10 which is also an antiproliferative peptide.

12. An anti-idiotype peptide of claim 10 which is conjugated to a second anti-idiotype peptide by a covalent linkage, a streptavidin-biotin linkage, or non-covalent binding association.

13. An anti-idiotype peptide of claim 12 wherein primary amino acid sequences of the anti-idiotype peptide and the second anti-idiotype peptide are identical.

14. An anti-idiotype peptide of claim 12 which inhibits proliferation of cells bearing a surface immunoglobulin superfamily molecule of an idiotype bound by the anti-idiotype peptide.

15. A kit for identifying anti-idiotypic and antiproliferative peptides by the method of claim 1, comprising a peptide library and an instruction manual.

16. A kit of claim 15, wherein the peptide library is a bacteriophage peptide display library comprising filamentous bacteriophage particles or filamentous bacteriophage genomes.

17. A therapeutic composition comprising an efficacious dosage of an antiproliferative peptide in a pharmaceutically acceptable form.

18. A therapeutic composition of claim 17, wherein the antiproliferative peptide is crosslinked by covalent linkage, biotin-streptavidin linkage, noncovalent binding association.

19. A therapeutic composition comprising a dimer of at least one anti-proliferative peptide.

20. A method for inhibiting the proliferation of lymphoma cells or lymphocytic leukemia cells, comprising delivering an inhibitory dose of a non-immunoglobulin antiproliferative peptide which specifically binds to an immunoglobulin superfamily molecule idiotype present on the lymphoma cells or lymphocytic leukemia cells.

21. A method of claim 20, wherein the non-immunoglobulin antiproliferative peptide is a dimer comprising two anti-proliferative peptide sequences.

22. A method of claim 21, wherein said two anti-proliferative peptide sequences are identical.

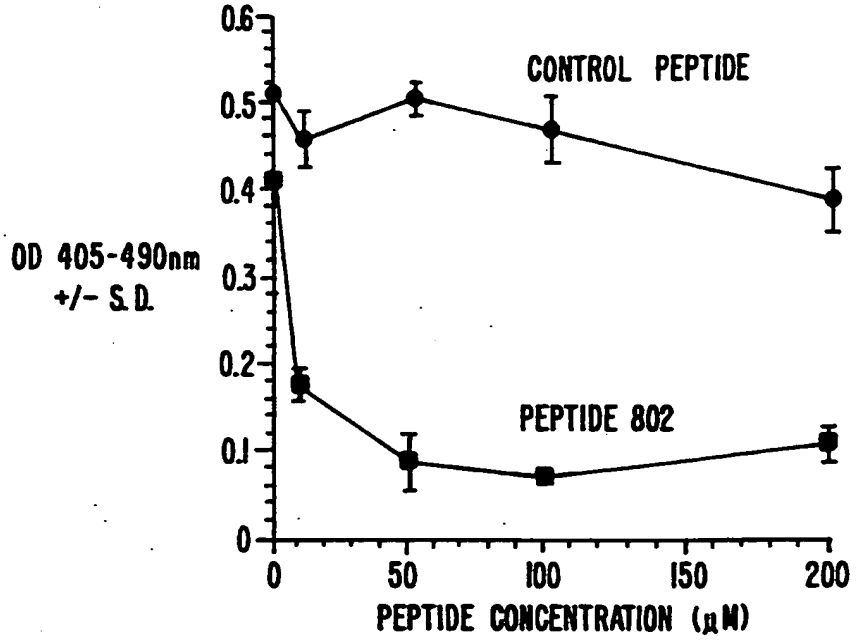
23. A method of treating a lymphoma or lymphocytic leukemia in a patient, comprising delivering a therapeutically effective dosage of a non-immunoglobulin antiproliferative

peptide which specifically binds to an immunoglobulin superfamily molecule idiotype present on the lymphoma cells or lymphocytic leukemia cells.

24. A method of claim 23, wherein the non-immunoglobulin antiproliferative peptide is a dimer comprising two anti-proliferative peptide sequences.

25. A method of claim 24, wherein said two anti-proliferative peptide sequences are identical.

26. A method of claim 20 or 23, wherein the non-immunoglobulin antiproliferative peptide is a dimer or tetramer.


0.6
0.5
0.4
0.3
0.2
0.1
0
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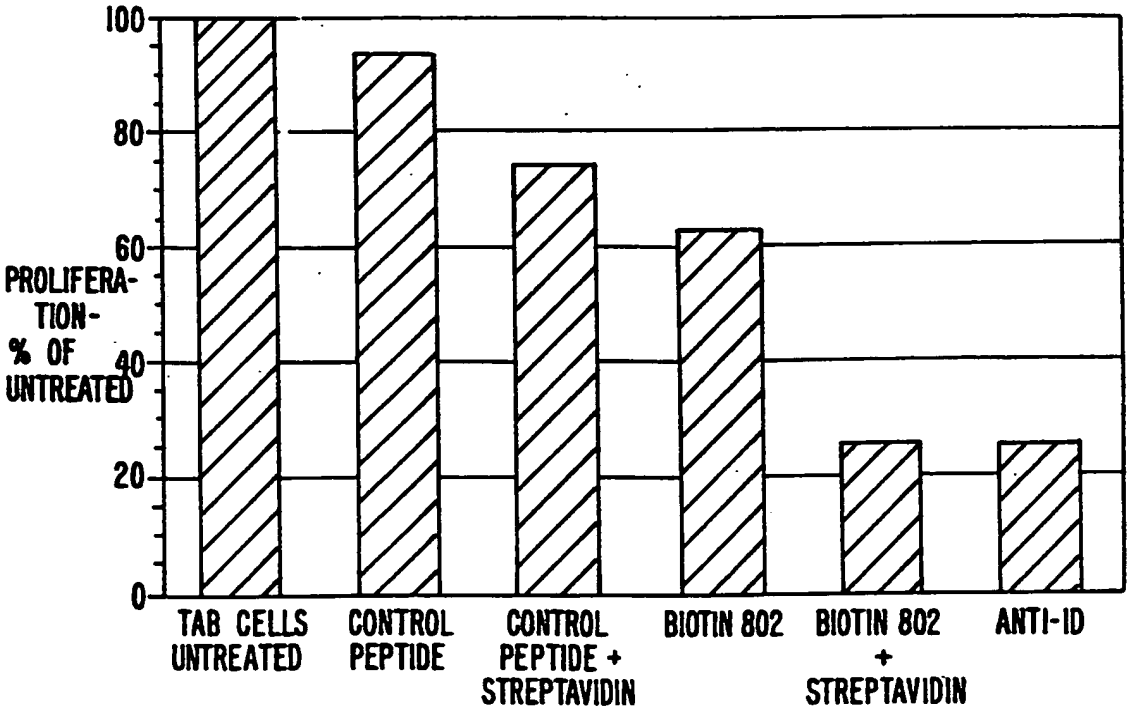
+/- S.D.
CONTROL PEPTIDE
PEPTIDE 802
0
50
100
150
200
PEPTIDE CONCENTRATION (μM)

FIG. 1.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80
60
40
20
0
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% OF
UNTREATED
TAB CELLS UNTREATED
CONTROL PEPTIDE
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BIOTIN 802
BIOTIN 802 + STREPTAVIDIN
ANTI-ID

FIG. 2.

Protein Tyrosine Phosphorylation in SUP-B8 Cells

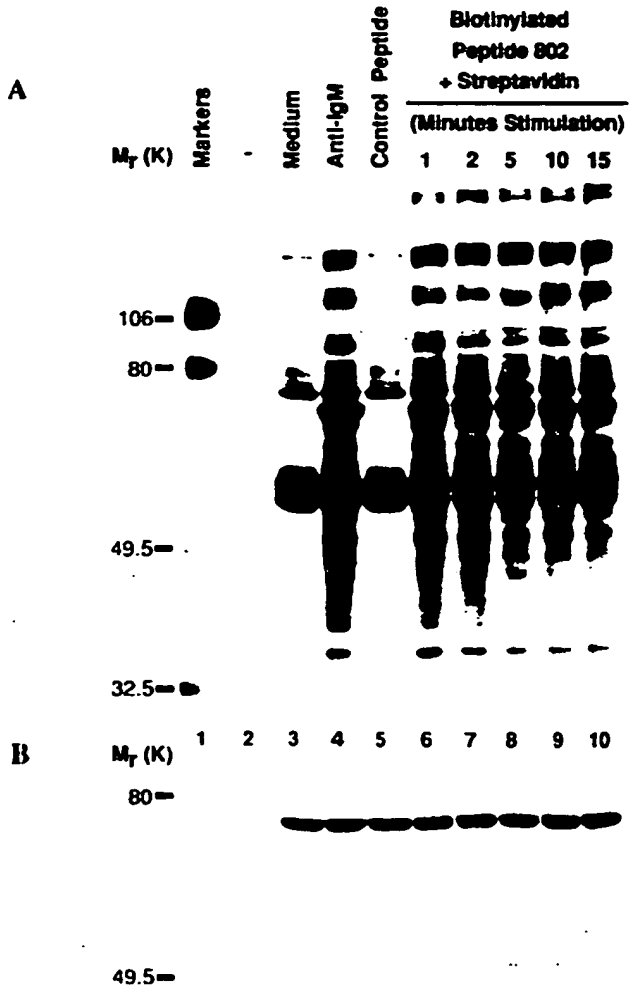
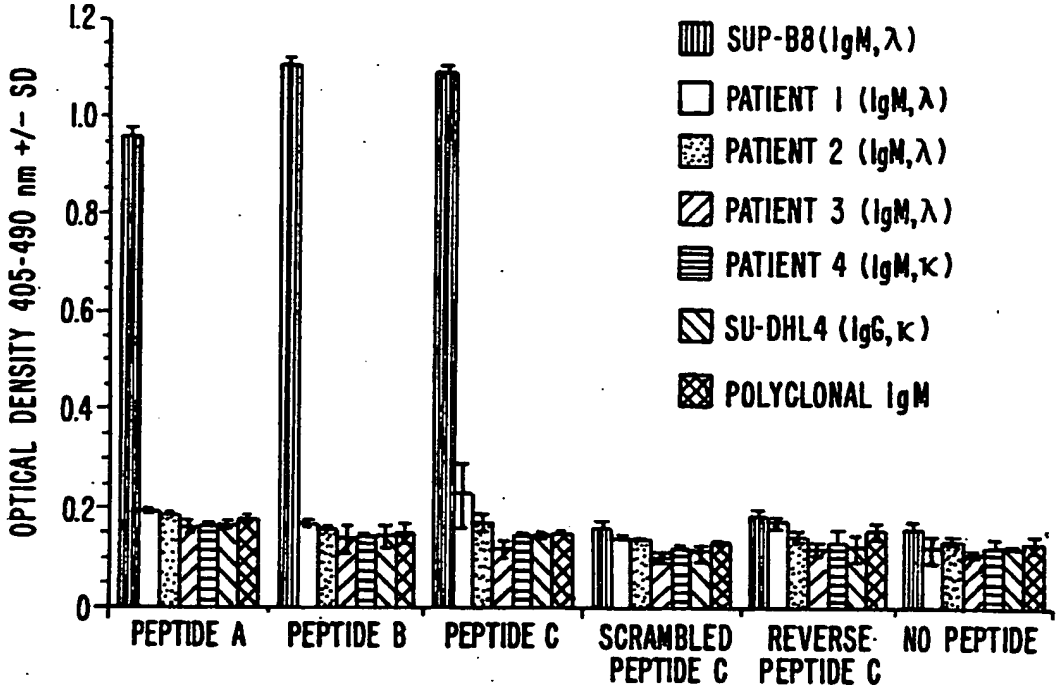


FIG. 3.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1.2
1.0
0.8
0.6
0.4
0.2
0
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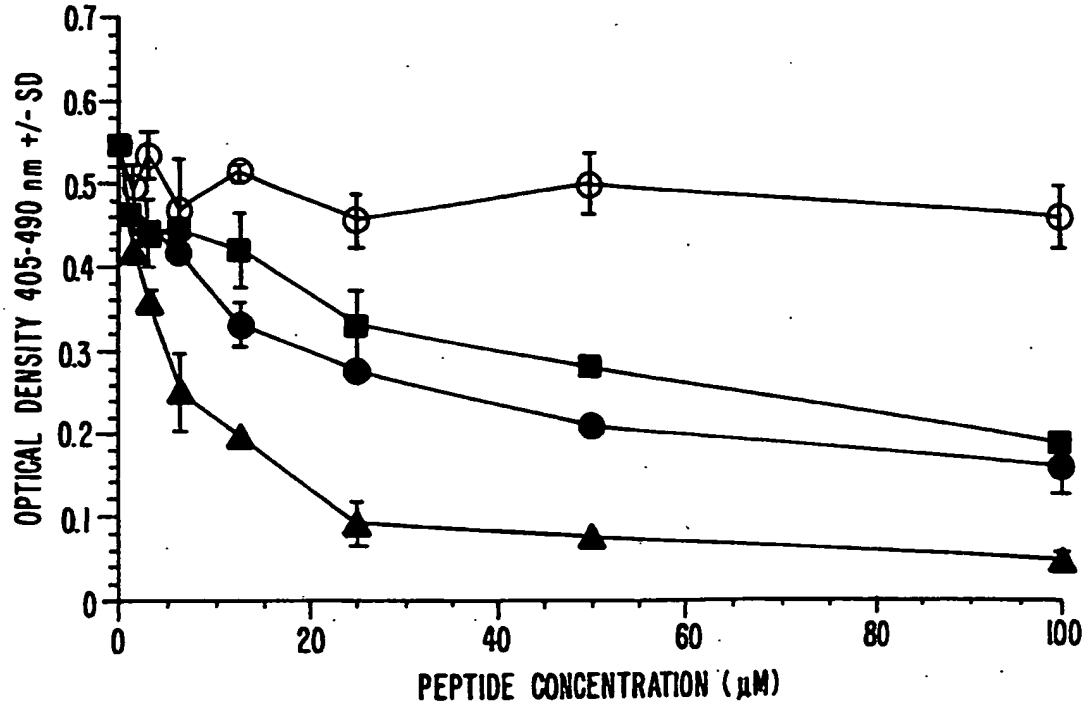
PATIENT 1 (IgM,λ)
PATIENT 2 (IgM,λ)
PATIENT 3 (IgM,λ)
PATIENT 4 (IgM,κ)
SU-DHL4 (IgG,κ)
POLYCLONAL IgM
PEPTIDE A
PEPTIDE B
PEPTIDE C
SCRAMBLED PEPTIDE C
REVERSE PEPTIDE C
NO PEPTIDE

FIG. 4.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0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
PEPTIDE CONCENTRATION (μM)

FIG. 5.

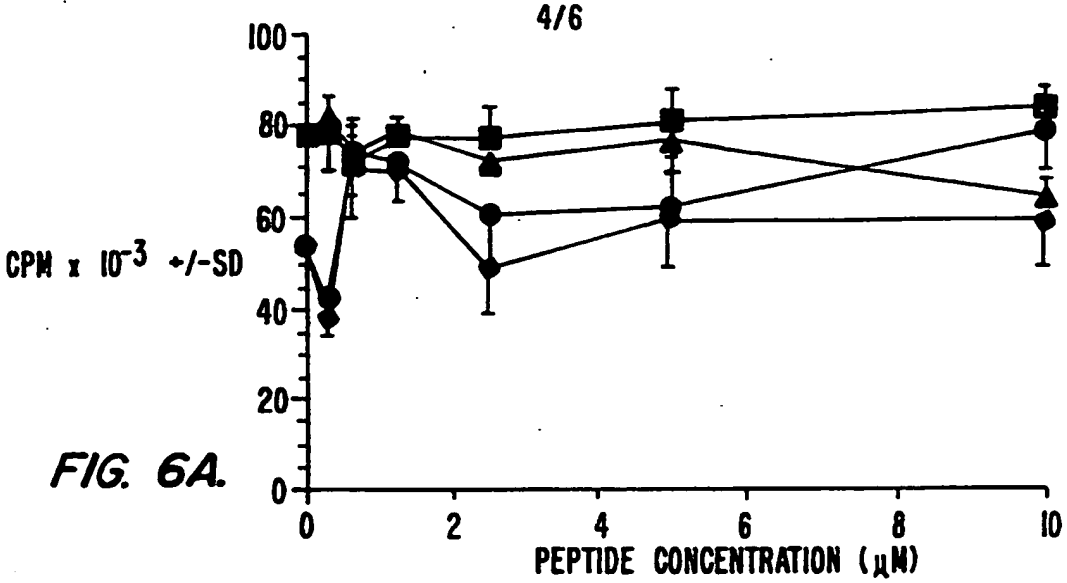

FIG. 6A.

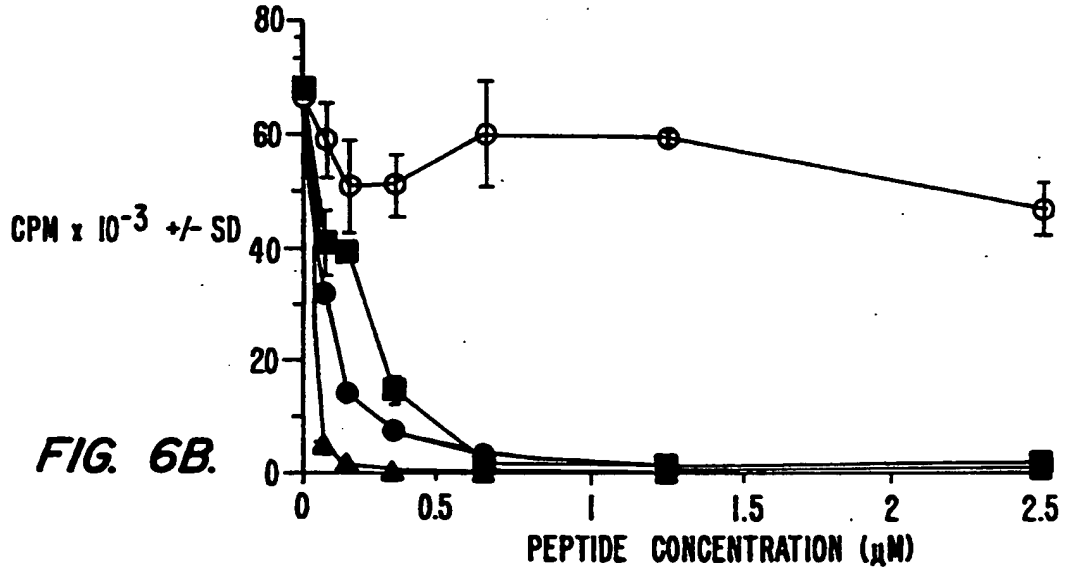

FIG. 6B.

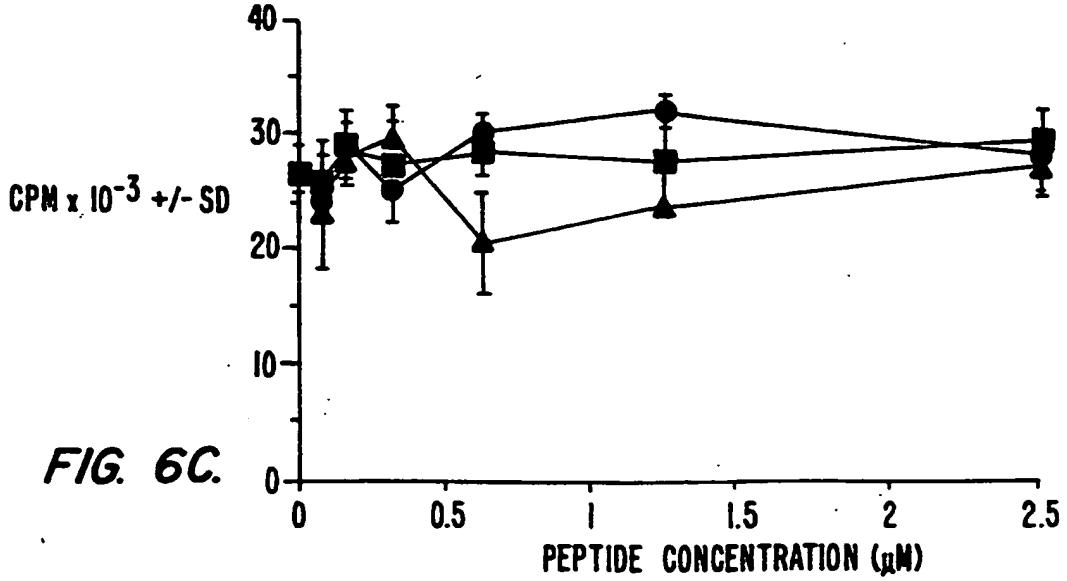

FIG. 6C.

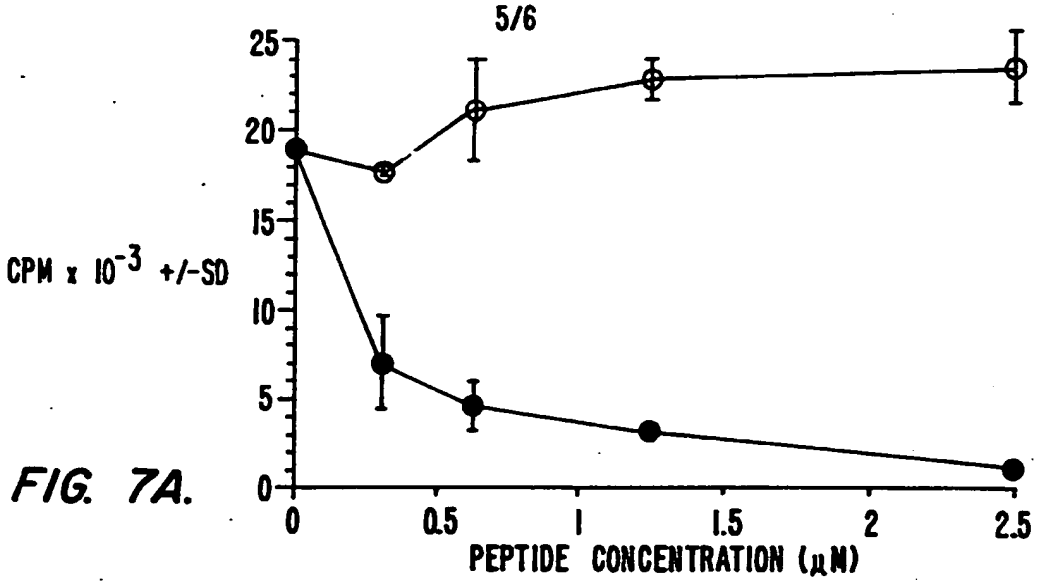

FIG. 7A.

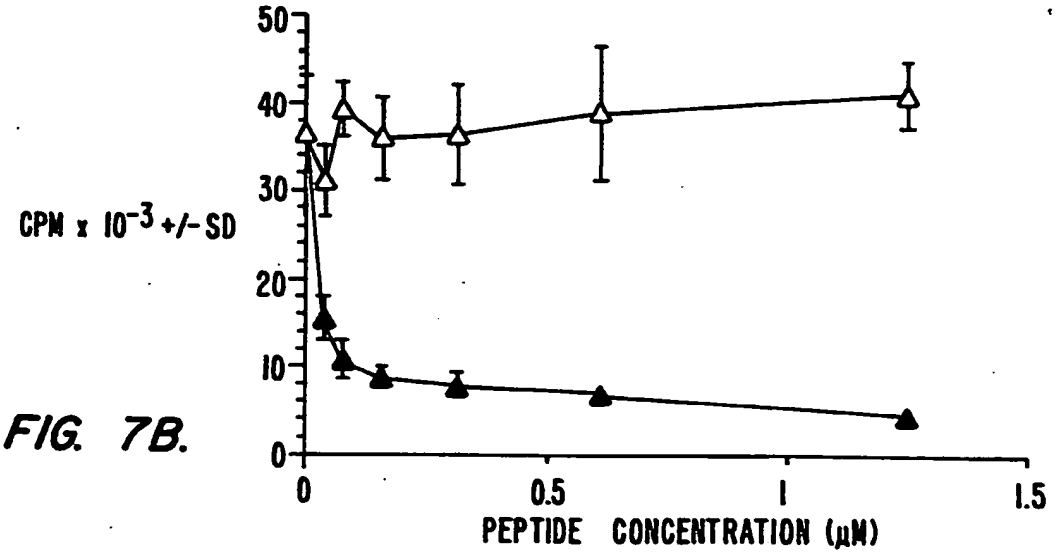

FIG. 7B.

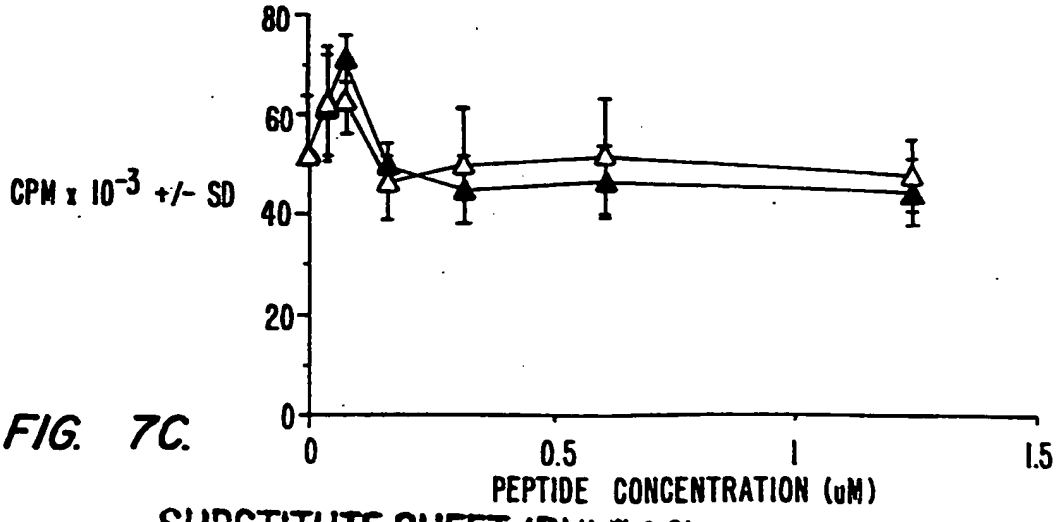

FIG. 7C.

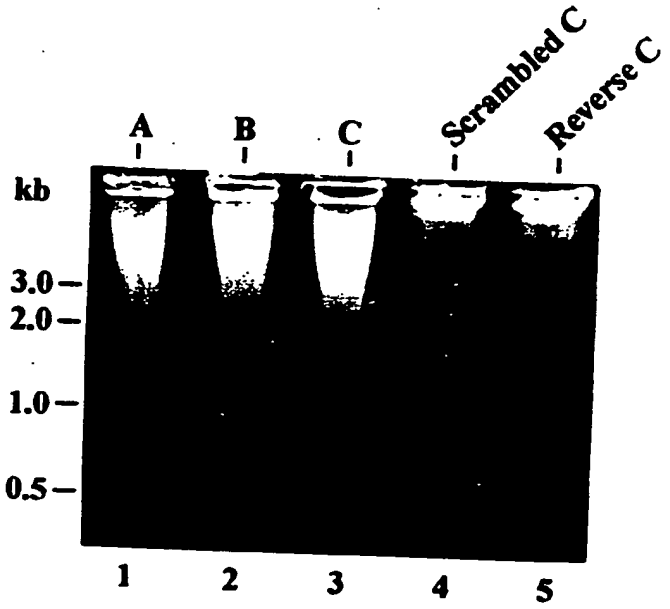

FIG. 8.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/01319

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12N 15/00; A61K 37/00
US CL : 435/6, 320.1; 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 69.1, 320.1, 965; 514/12; 935/60, 80, 81, 108

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	WO 91/19818 (DOWER ET AL.) 26 December 1991, pages 6-15 and 30-35.	15, 16 ---- 1-14, 17-26
Y	Hybridoma, Vol. 10, No. 2, issued 1991, A. J. T. George et al., "Monoclonal Antibodies Raised Against the Idiotype of the Murine B Cell Lymphoma, BCL1 Act Primarily with Heavy Chain Determinants," pages 219-227, see entire document.	1-14, 17-26
Y	Journal of the National Cancer Institute Monographs No. 10, issued 1990, R. Levy et al., "Therapy of Lymphoma Directed at Idiotypes," pages 61-68, see entire document.	1-14, 17-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 MARCH 1994	Date of mailing of the international search report 25 APR 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jill Warden for DONNA C. WORTMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*